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QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS: Some Observations on the Correlation in Identified Guinea-Pigs between their Responses to more than one Inoculation of Diphtheria Prophylactic.

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One of the first observations made when similar animals were given identical injections of an antigen was that the amount of antibody which subsequently appeared in their sera varied greatly from one individual to another. The variation in response was quite outside the normal range experienced with substances having a purely pharmacological effect. Comparable normal animals rarely differ by more than six-fold in their responses to drugs such as cocaine, digitalis and insulin (Trevan, quoted by Burn, 1950). On the other hand, the variation in antibody response to an inoculation of an antigen commonly varies fifty to one hundred-fold among small groups, and in some observations made on the antitoxin responses in human adults to injections of tetanus toxoid the extreme responses varied four thousand-fold; limits observed for two injections were 85 I.u./ml. serum to $<0{\cdot}02$ I.u./ml. in 1000 cases (unpublished work).

It is this gross variation in individual responses among the members of a group of similar animals to one or more injections of an antigen that renders quantitative work in immunity so unsatisfactory and accounts for the necessity to employ groups of animals and statistical devices when comparisons are being attempted.

The underlying physiological basis for these extreme variations in responsiveness to inoculations of a foreign protein is very obscure. We have, however, the observations of Carlinfanti (1948), who showed that individuals were relatively good or poor antibody producers to certain specific *groups* of antigens. This observation indicated that certain groups of antibodies were elaborated by closely related if not identical antibody-producing cells. That this might be the case is also implied by the fact that antibodies are not all associated with the same plasma protein fraction. For example, of the many γ-globulins present in human plasma (Cohn, Deutsch and Wetter, 1950), the iso-haemagglutinins are

primarily borne by the γ–1 globulin, and diphtheria antitoxin by the γ–2 globulin (Deutsch, Alberty, Gosting and Williams, 1947). Under exceptional circumstances, however, as during hyperimmunization in the horse, diphtheria antitoxin is associated initially with the γ-globulin fraction, and later predominantly with the β-globulin fraction. These two antitoxins show marked differences in avidity for toxin and in nitrogen per Lf of T.A.F. (Kekwick and Record, 1941). Recently Jager *et al.* (1950) claim to have shown hyperimmunized horse tetanus antitoxin to be spread over three globulins.

In our studies on the quantitative aspect of antitoxin production, it became of interest to observe the co-efficient of correlation in *identified* animals to more than one injection of diphtheria prophylactic, these observations being made in respect of both maximum titres developed and of resting level titres.

Without any guidance from specialized studies, it would be natural to assume that an individual which responded relatively well to a single inoculation would hold the same relative position following two.

Since evidence has been given to show that the antitoxin response to a primary injection of A.P.T. or P.T.A.P. is usually complicated by a superimposed secondary response effect (Holt, 1949), it was decided to measure the antitoxin titres not less than eight weeks after a primary inoculation. By adopting this procedure this effect of the superimposed secondary response leap was minimized and the values found represent the titres which remain substantially constant for some time—the base-line titre. The corresponding base-line titres following the second inoculations were measured 10–12 weeks after injection for the same reason. (N.B.—The circulating antitoxin due solely to the second response "leap," ten weeks after the second injection, amounts to approximately 6 per cent of the maximum titre attained, or 3 per cent after twelve weeks (Holt, 1950*b*).

Maximum second response titres were observed at the 12th and 14th day after the second injection, and the greater of the two values is reported. Occasionally the 14-day titre was appreciably greater than the 12-day one, and rarely significantly lower.

Experiment 1.

The primary object of this experiment was to observe whether the individual responses of the members of a group of animals to a small inoculum of adsorbed toxoid was any index of their responsiveness to subsequent intensive immunization.

Twenty guinea-pigs (weight 300 g.) were each inoculated subcutaneously on the ventral wall with 1·0 ml. of P.T.A.P., Lf 1, 1 mg. $AlPO_4$/ml., and their serum antitoxin titres were measured ten weeks later. Each animal was then given thirteen simultaneous inoculations of P.T.A.P. Lf 50, 7 mg. $AlPO_4$/ml. as follows :

0·5 ml. on each side of the neck,
4 × 0·5 ml. on each side of the body wall,
0·5 ml. on each hind leg,
2·0 ml. intraperitoneally.

Twelve weeks later their serum-antitoxin titres were again determined : The results are recorded in Table I, columns 1 and 2.

It will be seen that the response to a small inoculum of adsorbed toxoid

TABLE I.—*Antitoxin Titres in Guinea-pigs following more than one Inoculation of Diphtheria Toxoid.*

Animal No.	1*.	2.	3.	4.
1	0·07	8	100	12
2	0·2	7		
3	0·2	5	300	50
4	0·2	2		
5	0·2	5		
6	0·2	2·5	90	12
7	0·3	4	70	55
8	0·6	2·5	70	18
9	0·9	4	30	9
10	1·0	25		
11	1·0	6	200	40
12	1·25	9		
13	1·25	2		
14	1·25	4	90	70
15	1·25	4		
16	1·5	20	125	15
17	2·5	6		
18	2·5	3		
19	2·5	6	50	25
20	3·0			
Arithmetic mean	1·09	6·58	112·5	30·6
Geometric mean	0·69	5·08	92·3	24·2
Scatter	43 : 1	12·5 : 1	10 : 1	7·8 : 1

* For ease of direct comparison the guinea-pigs are placed here in order of increasing titre resulting from the first injection; the same has been done for the other groups.

1. Antitoxin titre 10 weeks after a primary inoculation of 1 Lf with 1 mg. $AlPO_4$.
2. Antitoxin titre 12 weeks after large multiple secondary stimuli (see text).
3. Maximum antitoxin titre following a large tertiary inoculation of formol toxoid.
4. Antitoxin titre 12 weeks after the large formol toxoid tertiary stimulus.

gave no indication of the animals' further capacity for antitoxin production. The following points may be noted:

(i) The animals which produced the highest second base-line titre of antitoxin, Nos. 10 and 16, were about average for the whole group as judged by their response to the first inoculation.

(ii) Two animals showed scarcely any change in their titre as a result of the very large second injection, Nos. 13 and 18, and these two were among those above the average in respect of the first injection.

(iii) The animal with the lowest titre (first response), No. 1, acquired a slightly greater than average titre after the second injection.

(iv) The scatter of titres among the group was reduced from 43 : 1 for the first injection, to 12·5 : 1 after the second.

Experiment 2.

In this experiment 36 guinea-pigs were given a preliminary subcutaneous inoculation of 0·5 ml. of P.T.A.P. Lf 2, 2 mg. $AlPO_4$/ml., and their serum-antitoxin titres were measured 8 weeks later. This time, instead of the second injection being massive as in the former experiment, much smaller inocula were administered. These comprised three different types of diphtheria prophylactic : (*a*) a duplication of the first inoculation, viz. P.T.A.P. Lf 2, 2 mg. $AlPO_4$/ml. (dose 0·5 ml. subcutaneously), (*b*) the same material " neutralized " with 2u/ml. of native guinea-pig antitoxin and stored for ten weeks at 5° before use, this to

TABLE II.—*Antitoxin Titres in Guinea-pigs following First and Second Injection of Diphtheria Prophylactic.*

(All animals equally primed with 1 Lf with 1 mg. $AlPO_4$).

Animal No.	Titre 8 weeks after primary.	Titre 10 weeks after second.	
1	0·02	1·25	
2	0·04	0·5	
3	0·15	—	
4	0·3	0·6	
5	0·3	0·6	
6	0·3	1·25	
7	0·8	1·5	
8	0·8	—	*Second stimulus :* 1 Lf with 1 mg. $AlPo_4$.
9	0·8	2·5	
10	1·0	1·75	
11	1·25	7·0	
12	1·25	1·0	
Scatter	62·5 : 1	14 : 1	
Arithmetic mean	0·58	1·80	
Geometric mean	0·34	1·29	
13	0·01	1·75	
14	0·07	1·75	
15	0·08	4·0	
16	0·1	—	*Second stimulus :* 1 Lf with 1 mg. $AlPO_4$ " neutralized " with guinea-pig antitoxin.
17	0·3	1·0	
18	0·3	0·2	
19	0·3	1·25	
20	0·4	1·25	
21	0·4	1·25	
22	0·6	2·5	
23	0·7	1·25	
24	0·7	2·5	
Scatter.	70 : 1	20 : 1	
Arithmetic mean	0·33	1·70	
Geometric mean	0·21	1·39	

TABLE II.—*cont.*

Animal No.	Titre 8 weeks after primary.	Titre 10 weeks after second.	
25	0·3	0·4	
26	0·3	1·5	
27	0·3	2·5	
28	0·5	4·0	*Second stimulus :* 25 Lf with 1 mg. $AlPO_4$.
29	0·5	2·5	
30	0·6	5·0	
31	0·7	7·0	
32	0·7	2·5	
33	1·0	4·0	
34	1·0	2·5	
35	1·25	1·75	
36	1·25	2·5	
Scatter.	4·2 : 1	17·5 : 1	
Arithmetic mean	0·70	3·01	
Geometric mean	0·62	2·49	

Arithmetic mean (total) 0·54
Geometric mean (total) 0·35

t., Animals 1–12/13–24 (Primary) = 1·70, P = 0·1
„ 1–12/25–36 „ = 0·76, P = > 0·4
„ 1–12/13–24 (Secondary) = 0·151, P = > 0·8
„ 1–12/25–36 „ = 1·53, P = >0·1

observe whether the depressing effect of antitoxin on the antigenicity of toxoid administered as a primary stimulus held equally true for a second stimulus, and (*c*) P.T.A.P. Lf 50, 2 mg. $AlPO_4$/ml. (dose 0·5 ml.). Twelve animals were used for each of the three different secondary stimuli. The results obtained are shown in Table II. Again the same irregular results were obtained ; the very low initial serum antitoxin titres increased to substantial values ; the moderate initial titres increased very irregularly, and the serum titre of one of the animals (No. 12) showing the greatest response 8 weeks after the primary inoculation was *lower* 10 weeks after the second injection.

Despite the marked irregularity of response to one and two doses of diphtheria prophylactic among these 52 animals, it was thought there might be some larger measure of correlation between initial resting level titres and *maximum* secondary response titres.

It had been found that when a subgroup of primed guinea-pigs were given daily inoculations of 1 Lf of formol-toxoid for 25 days consecutively, the secondary response antitoxin titre curve reached its maximum value not later than 3 days after a control subgroup given one dose of 1 Lf only (Holt, 1950*a*). It was concluded that the first 3 doses of 1 Lf of toxoid were adequate to " fire off " all the antitoxin potential of the animals employed. In order to be certain, however, that all the " potential " of our primed animals was called into play, two secondary-stimulus dosages were employed ; (*a*) to inoculate each animal daily

for 3 days, with 4 Lf amounts of formol-toxoid, and (*b*) to inject an amount of toxoid equivalent to the calculated sum total of circulating antitoxin present in the animal's blood stream. This latter was done by titrating each animal's serum for antitoxin, weighing the animal, and assuming the plasma volume to be 5 per cent v/w.

Three maximum secondary response experiments were carried out:

Experiment 3.

Ten guinea-pigs were given a primary inoculation of 0·5 ml. of P.T.A.P. Lf 2, 2 mg. $AlPO_4$/ml. administered subcutaneously. Ten weeks later their serum antitoxin titres were measured, and each animal was then given 4 Lf of formol toxoid daily for 3 days. The maximum antitoxin titres developed were measured as before; the results are shown in Table III.

TABLE III.—*Maximum Second Response Antitoxin Titres in Guinea-pigs.*

Animal No.	Serum titre 10 weeks after primary inoculation of 1 Lf and 1 mg. $AlPO_4$ (u./ml.).	Maximum titre following a secondary stimulus of 3 daily injections of 4 Lf formol toxoid (u./ml.).
1	0·02	15
2	0·2	20
3	0·25	6
4	0·4	40
5	0·4	8
6	0·6	10
7	0·6	17
8	1·25	15
9	1·25	25
10	2·5	17
Arithmetic mean	0·75	17·3
Geometric mean	0·43	15·1
Scatter	125 : 1	6·7 : 1

Experiment 4.

Four groups of guinea-pigs were primed with (*a*) 1 Lf toxoid with 1 mg. $AlPO_4$, (*b*) two simultaneous inoculations of 0·5 Lf toxoid with 1 mg. $AlPO_4$, (*c*) three simultaneous inoculations of 0·33 Lf toxoid with 1 mg. $AlPO_4$, and (*d*) four simultaneous inoculations of 0·25 Lf toxoid with 1 mg. $AlPO_4$, all administered subcutaneously. Twelve weeks later their individual antitoxin titres were measured. Each animal was then inoculated subcutaneously with the formol-toxoid equivalent of its calculated total plasma antitoxin. The maximal antitoxin titres were then measured. The results obtained are recorded in Table IV.

Experiment 5.

To contrast with the above animals, each of which had received a primary inoculation only, ten of the hyperimmunized animals recorded in Table I were selected. Each animal was bled 12 weeks after its large multiple second stimulus,

TABLE IV.—*Maximum Second Response Titres in Groups of Guinea-pigs.*

Primary inoculation for Group A : 1 Lf with 1 mg. $AlPO_4$.
" " " B : Two inoculations each 0·5 Lf with 1 mg. $AlPO_4$.
" " " C : Three inoculations each 0·33 Lf with 1 mg. $AlPO_4$.
" " " D : Four inoculations each 0·25 Lf with 1 mg. $AlPO_4$.

Second stimulus : The formol toxoid equivalent of the calculated total plasma antitoxin, administered 12 weeks after primary inoculation.

Individual Antitoxin Titres, u./ml.

No.	*Series A.*		*Series B.*		*Series C.*		*Series D.*	
	Primary.	Secondary.	Primary.	Secondary.	Primary.	Secondary.	Primary.	Secondary.
1	0·1	4	0·2	12	0·2	15	0·2	17
2	0·1	10	0·3	1·25	0·4	20	0·4	17
3	0·4	40	0·4	55	0·4	50	0·5	22
4	0·6	40	0·4	10	0·5	70	1·0	70
5	0·7	12	0·5	70	0·5	25	1·25	60
6	0·7	9	0·5	17	0·8	40	1·25	50
7	0·7	50	0·5	17	0·9	40	1·25	18
8	0·8	12	0·5	25	1·25	70	2·0	40
9	2·0	60	1·25	25	1·25	55	2·5	250
10	4·0	80	1·25	50				
11			1·25	25				
Arithmetic mean	1·01	31·7	0·64	27·90	0·69	42·8	1·15	60·4
Geometric mean	0·89	21·35	0·54	19·20	0·59	37·0	0·90	39·61
Scatter	40 : 1	20 : 1	6 : 1	56 : 1	6 : 1	4·7 : 1	12·5 : 1	14·7 : 1

t., Series A/Series D (Primary) = 0·313, P = > 0·7.
" " (Secondary) = 1·15 P = 0·25.

its antitoxin titre measured and then was given subcutaneously the formol toxoid equivalent of its calculated total plasma antitoxin. The maximum antitoxin titres were measured ; the values obtained are recorded in Table I, columns 2 and 3.

The serum antitoxin titres of this selected group were measured again 12 weeks after the large formol toxoid tertiary stimulus (Table I, column 4). This was to observe, even with the few animals used, whether there was any direct correlation between their three base-line titres. It will be noted that again there occurred the same irregularity between the second and third base-line titres.

In contrast, however, with the consistently irregular results obtained for base-line titres, when the maximum secondary response titres were measured, a definite but not perfect correlation may exist between the and the pre-existing base-line titres. This is clearly shown in Table IV, but is absent in Table III or Table I, column 2 and 3. In Table IV the coefficient of correlation is + 0·623 for 37 df, for which a P value of <0·001 is obtained, whereas in the others P=>0·1 (see Appendix).

DISCUSSION.

The above experiments employing identified animals, and observing their responses to a variety of secondary stimuli, reveal a marked irregularity in behaviour. Expressed statistically, the coefficient of correlation between the response to an efficient primary inoculation and a well-spaced second injection (base-line titres) is not significantly different from zero at the 5 per cent level.

On the other hand, when the maximum second response titres are measured, despite the wide range of values, a high coefficient of correlation was obtained ($P = <0{\cdot}001$) when the stimulus *is the toxoid equivalent of the calculated total circulating antitoxin for each animal used*, and the animals are not hyperimmunized.

Although the distribution of the log of responses among the members of a population to one inoculation is normal, and the increases in base-line titres following a second inoculation are so irregular, it is noteworthy that the new distribution of values (log. titres) also is normal. Furthermore it is found that the logs. of the *maximum* titres obtained (Table IV) are also normally distributed (see Appendix).

When a small number of animals were followed over an intensive three dose system (Table I) using massive secondary and tertiary stimuli, remarkable differences in behaviour appeared. Some animals, as a result of the large tertiary stimulus, produced very high titres indeed, 200–300 u./ml., and others much lower ones, but whereas most showed a marked decline in titre over the subsequent 10 weeks, in two animals the antitoxin titre remained substantially unaltered from its maximum value (Table I, columns 3 and 4, animals 7 and 14). In contrast animal 16 had a lower titre 12 weeks after the large tertiary stimulus than immediately before.

Although the modern tendency is to ascribe to certain mesenchymal cells the function of elaborating serum globulins, and *ipso facto* antitoxic globulins, the data presented above reveal how very complex is the physiology of antitoxin production. It is shown that animals which respond equally well to a small primary inoculation of diphtheria toxoid, when further stimulated rapidly reveal large differences in their capacity to make and maintain circulating antitoxin. Furthermore, in an attempt to convert every potential antitoxin-producing cell in the guinea-pig to one making antitoxin (Table I), it was found that on further stimulation some animals responded by increasing their base-line titre of antitoxin by as much as eighteen times, and others remained almost unaffected.

Since under normal circumstances the known plasma protein fractions are approximately uniform quantitatively among the individuals of a given species (Moore, 1946, Martin and Morris, 1949; Moore, Du Pan and Buxton, 1949), some controlling mechanism must operate to maintain this proportionality. A disturbance in this mechanism during hyperimmunization must occur, as it has been shown, notably in the horse, that a state of hyperglobulinaemia develops (His and Atkinson, 1901; Ledingham, 1907; MacFarlane, 1935; Kekwick and Record, 1941). But, as a consequence of the hyperimmunization, the total increase in the plasma globulin is in gross excess of the precipitable antibody produced. Furthermore, the increase in plasma globulin may continue without a further rise in antitoxin titre during the course of successive inoculations of toxoid (Kekwick and Record, 1941).

One possible explanation for the irregular results obtained in the above maximum second-response experiments (Table IV, where the coefficient of correlation is high and the titres showed a tendency to increase one hundred-fold) could be that the apparent differences in responsiveness of the animals to the comparable second stimulus lies in their "failure" to make only antitoxic globulin as a result of the stimulus, the tendency to excessive hyperglobulinaemia being limited by the mechanism regulating the normal plasma protein composition.

In keeping with this we have the observation that as the amount of plasma globulin increases during hyperimmunization, the absolute concentration of albumin falls. This fall in the concentration of albumin would rectify the tendency for the hyperglobulinaemic plasma, *per se*, to acquire an abnormal differential osmotic pressure.

In respect of the irregular increases in the base-line titres as a consequence of two or more spaced inoculations the problem is different, although not completely unrelated to the limitation of maximum response. When the animals in Experiment 1 (Table I) were given a very massive second stimulus—believed sufficient to ensure that every potential antitoxin-producing cell was adequately stimulated—a great increase in the base-line titre of most of the animals' sera was brought about. Yet, when those same animals received a third stimulus, the average base-line titre was increased from 6 u./ml. to 30 u./ml. (columns 3 and 4).

If the not unreasonable assumption is made that the different plasma proteins are elaborated by different cells, and that the *relative number of each of these cells varies in different individuals* of the same species and of the same weight, then a first interpretation of the above experimental results may be attempted. Since Carlinfanti's observations imply that one group of antibody-producing cells will react only to antigens of a similar type, when therefore the members of a group of similar animals, each containing a different number of the appropriate antibody-producing cells, are given an identical inoculation, the inoculum will be spread over many cells, reactive and non-reactive. Whence, for the given inoculum, those animals possessing a greater number of the reactive cells will exhibit the greater antibody response.

In the above observations, where there occurred an increase in the base-line titres following a tertiary stimulus, and where the second injection would seem to have been more than adequate to stimulate every antibody-producing cell in the animal employed (Table I), it is suggested that the most likely way for the increase of base-line titre to have occurred is by *hyperplasia of previously primed cells*. And since the increase of antitoxin titre persists, it must follow that there occurs a relative, but far from absolute, specific hyperplasia among all the globulin-producing cells. The overall hyperplasia, it is suggested, may also be limited, but to a varying degree in different individuals, by the mechanism normally controlling the proportions of the plasma proteins.

SUMMARY.

Guinea-pigs were given a uniform primary inoculation of diphtheria prophylactic. Groups of these primed animals were then given very different secondary stimuli, and the individual base-line antitoxin titres again measured. Marked irregularity in relative response to the first and second injections was found: the coefficient of correlation between the first and second base-line titres was not significantly different from zero at the 5 per cent level.

All the animals which were relatively very poor antitoxin producers to the primary inoculation responded well to the second stimulus, and fall into the average values for their group.

A high coefficient of correlation was found between initial base-line titres and *maximum* secondary response titres, when the stimulating dose of toxoid was

equivalent in each case to the calculated total circulating antitoxin, and the animals were not hyperimmunized, but very low when each animal received a large but uniform inoculation.

It has been shown by Carlinfanti and Cavalli that the distribution of antibody titres among a population which had received one inoculation was normally distributed when plotted on a log. basis ; it is now found, in guinea-pigs, that the distribution of maximum second responses and second base-line titres is similarly normally distributed.

Grateful thanks are due to Dr. L. Cavalli, of the Department of Genetics, Cambridge, for most valuable advice in the statistical analyses.

APPENDIX.

A Statistical Examination of the Results.

It is required to know the degree of correlation of antitoxin responses in identified similar animals to more than one inoculation of diphtheria prophylactic.

A preliminary requisite was to observe if the logs. of the secondary response titres are normally distributed, as are the primary (Carlinfanti and Cavalli, 1945 ; Carlinfanti, 1948). By taking advantage of Fisher's Table XX for this purpose (Fisher and Yates, 1948), the second response titres, both maximal and new base-line, are found also to have a normal distribution (see Figs. 1 and 2).

The standard statistical correlation equation seemed adequate for the purpose, but was slightly increased in accuracy by using the log. of the individual titres, instead of the more usual arithmetic number found in the experiment.

Whence :

$$r = \sqrt{b\ b'} = \frac{S\,(x - \bar{x})\,(y - \bar{y}).}{\sqrt{S(x - \bar{x})^2\,S(y - \bar{y})^2}}$$

Resting-level Titres.

TABLE I : Columns 1 and 2.

Correlation between resting-level titres from a small primary inoculation, and the resting-levels following a massive second stimulus.

$$r = 0{\cdot}139 \text{ for } 17\text{df}.$$

TABLE II.

Correlation between resting-level titres from a uniform small primary inoculation and that following three small but different types of second stimulus.

Animals 1–12,	$r = +\ 0{\cdot}511$	df = 8
Animals 13–24,	$r = -\ 0{\cdot}177$	df = 9
Animals 25–36,	$r = +\ 0{\cdot}399$	df = 10
Pooled data	$r = -\ 0{\cdot}062$	df = 31

In no case is the coefficient of correlation significantly different from zero at the 5 per cent level.

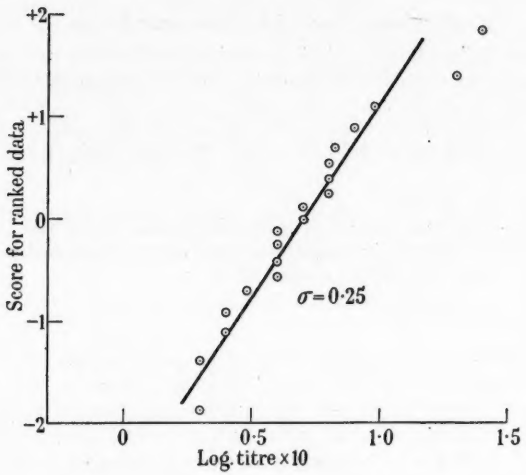
+2
+1
0
−1
−2
Score for ranked data
σ=0·25
0
0·5
1·0
1·5
Log. titre x10

FIG. 1.

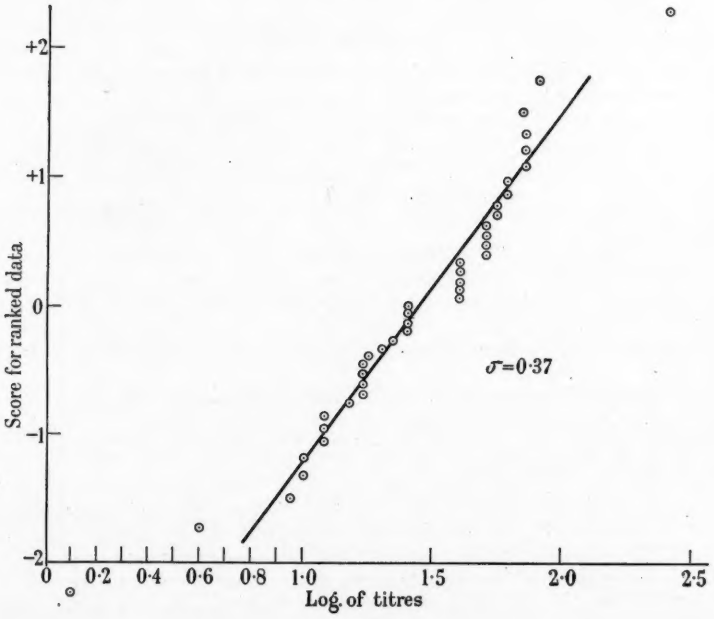
+2
+1
0
−1
−2
Score for ranked data
σ=0·37
0
0·2
0·4
0·6
0·8
1·0
1·5
2·0
2·5
Log. of titres

FIG. 2.

Maximum Secondary Response Titres.

A. Correlation between pre-existing resting-level titres and maximum titres following a stimulus comprising three daily inoculations of 4 Lf formol toxoid.

TABLE III.

$r = + 0{\cdot}14$ $\quad df = 8, P = \geqslant 0{\cdot}1$
$\quad (P, 0{\cdot}1 = 0{\cdot}549)$

B. Correlation between pre-existing resting-level titres and maximum titres following a stimulus comprising that amount of formol-toxoid (Lf) equivalent to the calculated total circulating antitoxin.

TABLE I: columns 2 and 3.

$r = 0{\cdot}278$ $\quad df = 8, P = > 0{\cdot}1$
$\quad (P, 0{\cdot}1 = 0{\cdot}549)$

TABLE IV

Series.	r.	df.	P.
A	+ 0·755	8	<0·02
B	+ 0·469	9	>0·1
C	+ 0·685	7	0·05
D	+ 0·724	7	<0·05
Pooled data	+ 0·623	37	<0·001

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THE MOVEMENTS OF TOXIN IN THE NERVOUS SYSTEM IN EXPERIMENTAL TETANUS IN RABBITS.

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In studies on the pathogenesis of tetanus, investigators have frequently made use of experiments in which the toxin was injected directly into some large nerve trunk. Though such procedures are clearly artificial, in that they fail to simulate any natural infection and intoxication, they are justified by the ample evidence that tetanus toxin injected into the muscles of a limb can be recovered some hours later from the nerve trunks that supply them (Bruschettini, 1892; Fildes (for literature), 1929; Baylis *et al.*, 1950). The results of such intraneural inoculations depend on the quantity of toxin injected: if small, local tetanus only develops; if large, local tetanus appears more rapidly, and is succeeded by manifestations of generalized intoxication and death.

Most of the early investigators of the pathogenesis of tetanus agreed in believing that the toxin was carried from its site of formation or inoculation centripetally along motor nerve trunks until it reached the spinal cord, whose vulnerable structures it injured, and in whose tissue spaces it ascended to successively higher levels until finally it caused a fatal intoxication of the brain stem (see Friedemann, Hollander and Tarlov, 1940, for literature). Shortly before the war this interpretation was challenged by Abel and his collaborators in a long series of papers (see Abel *et al.*, 1935*a*, *b* and *c*, 1938; Harvey, 1939; Firor, Lamont and Shumacker, 1940). In these they recalled and supported the earlier belief of Brunner (1892) and Goldscheider (1894) that the toxin had a dual locus of action; it brought about the spastic features of local tetanus through changes in the excitability of the axon terminals in the regional muscles; and it caused its generalized signs of central nervous system intoxication after it had been carried to these structures by circulating blood and not along pathways in nerve trunks.

In the course of their comprehensive discussion of this question Abel and his associates rejected the evidence for the belief that even intraneurally injected toxin spreads to the central nervous system by centripetal movement along motor nerve trunks on three grounds: firstly, that any such inoculation of toxin inevitably leads to contamination of surrounding structures by leakage at the site of puncture, so that no conclusion can be drawn safely as to whether the action of the toxin is local or central; secondly, the attempt to create a barrier to the ascent of toxin by a proximal intraneural injection of specific antitoxin is equally suspect for the same general reason—the escaping antitoxin spreads from the needle track and neutralizes the toxin while the latter is still

within the muscular structures of the limb ; and thirdly, that any attempt to obstruct the ascent of the toxin by mechanical measures, as by prior sclerosis of the nerve trunk proximally, so damages the nerve that any later absence of spasticity distally is to be attributed to paralysis of the limb rather than to prevention of central intoxication.

We have discussed the first of these criticisms in a paper (Wright *et al.*, 1950) which dealt with experimental tetanus intoxications of the brain stem. There we showed that after inoculation of toxin into various motor cranial nerves, manifestations of tetanus appeared subsequently in structures that were so far removed from the site of injection that no possibility of their contamination could arise. The experiments described in the present paper, in all of which tetanus toxin has been injected into the sciatic nerves of rabbits, have been designed to throw light on the validity of the second and third of Abel's criticisms. They have been planned on the following three lines : (i) to determine whether tetanus antitoxin when injected intraneurally at a symmetrically placed site *in the opposite limb* can exert any modifying influence on the severity of the subsequent tetanus (perhaps by creating an " immunological blockage " of the supposed ascending pathway of the toxin at the level of the lumbar enlargement ; (ii) to determine whether a prior sclerosis of the connective tissues of the nerve can mitigate the subsequent course of the intoxication without causing serious paresis of the limb ; and (iii) to attempt to trace any movements in both the nerve trunk and cerebrospinal axis of an intraneurally injected protein (of about the believed molecular size of the toxin) which had been rendered identifiable by labelling with radioactive iodine.

MATERIALS AND METHODS.

(i) *Toxin.*—The preparation used was the same as that employed previously (Wright *et al.*). Its potency had decreased slightly since it was first made, but not sufficiently to require any material change in dosage or to affect the course of the intoxication. The dried toxin was kept in a vacuum desiccator, and fresh solutions in sterile saline were made immediately before each experiment ; they were usually injected within 15 minutes of preparation. In those experiments in which it was added to the radio-iodinated serum proteins, however, the toxin powder was dissolved in the solution 4 hours before the end of its dialysis. It was found that dialysis against saline in the cold for periods up to 24 hours had little effect on the potency of the toxin for rabbits. From the few rough experiments made, the intravenous LD_{50} for rabbits was estimated to be about 100 μg. per kg.

(ii) *Animals and operative procedures.*—Albino rabbits, almost all weighing between 2 and 3 kg., were used throughout. Before intraneural injections the animals were lightly anaesthetized with " veterinary Nembutal " (25 mg. per kg.) given intravenously, and surgical anaesthesia then obtained with open ether. The sciatic nerve was rapidly exposed by aseptic technique for 3 to 4 cm. of its course, and rendered lightly tense by retraction of its lower end with a blunt metal hook. The toxin, dissolved in 0·04 ml. saline, was injected into the nerve in the mid-femoral region ; subsequent anatomical dissections showed that this site of inoculation was about 7 to 8 cm. distal along the trunk from the point of emergence of the main sciatic nerve roots from the spinal cord. Injections

were made with a 1 ml. tuberculin syringe fitted with a No. 27 gauge needle, and controlled by a micrometer-head screw to deliver small volumes with accuracy. Occasionally, towards the end of the inoculation, a small bleb appeared beneath the perineurium, but in most instances the injection merely resulted in a slightly fusiform distension of the nerve trunk.

(iii) *Antitoxin.*—The antitoxins used were either a Wellcome refined antitoxic globulin preparation, or a crude antitoxic horse serum kindly supplied to us by Dr. C. L. Oakley.

(iv) *Nerve sclerosis.*—Sclerosis of the neural connective tissues was effected by the intraneural injection of 0·04 ml. of ethanolamine oleate (Injectio Aethanolamine oleatis, B.P.) divided between the two main trunks of the sciatic nerve in the upper femoral region. In one rabbit the same volume of a quinine-urethane solution (Injectio quininae et urethani, B.P.) was given instead. Both solutions gave similar sclerosing effects. An interval of 14 to 21 days was allowed to elapse between the inception of sclerosis and the later distal injection of the toxin solution.

(v) *Preparation of radio-iodinated serum proteins.*—Fresh rabbit serum (0·1 ml.) was ammoniated with 5N NH_4OH (0·05 ml.). To this was added a mixture of I-KI solution (0·10 ml.), and the carrier-free radioactive iodine containing 200 μC. of I^{131}. The above I-KI solution contained 1·23 mg. I_2 and 1·57 mg. KI per ml. The volume of the radioactive iodide solution as supplied from A.E.R.E. varied in different consignments from 0·23 to 0·50 ml., so that the final volumes of the different preparations of the iodinated protein solutions varied accordingly. These variations were reflected in differences in the radioactivity of the final protein-toxin solutions used. After the addition of the I-KI mixture the serum solution was allowed to stand at room temperature for 60 minutes; it was then dialysed in the cold room through a cellophane membrane against hourly-changed litre volumes of 0·9 per cent NaCl solution. Dialysis, which usually lasted 5 or 6 hours, was terminated when the escape of uncoupled I^{131} had proceeded so far that a 10 ml. sample of the dialysate saline gave a count of less than twice the background in a liquid M.6 counter.

(vi) *Removal and fixation of tissues.*—Those rabbits that survived the toxin injection for 16 hours were given 0·2 ml. heparin solution (Liquemin Roche, 5000 units per ml.), followed by nembutal-ether anaesthesia as above. They were then killed by exsanguination. Immediately after death the aorta was cannulated through the left ventricle and the right auricle opened. The carcass was then perfused under low pressure with 500 ml. 0·9 per cent NaCl solution, immediately followed by 500 ml. absolute industrial methylated spirit. This treatment removed almost all the blood and rendered all tissues much firmer; it enabled the brain, spinal cord and sciatic nerves to be removed without damage. After removal, these parts of the nervous system, still in continuity, were immersed in a tray of absolute alcohol for further fixation. The rabbits that had died before 16 hours as a result of tetanus intoxication were similarly perfused with saline and alcohol, but owing to partial coagulation of the blood this was less successful. In these animals, therefore, after exposing the required parts *in situ*, the tissues were fixed with spirit before removal. To exclude any possible spurious contribution made by residual blood to the counts given by the samples of the nervous tissues, portions of dorsal muscle were taken from poorly exsanguinated animals; none was found to be significantly above the background count.

(vii) *Estimations of radioactivity of samples.*—All measurements of radioactivity of tissue samples were made on a single G.M. counter tube (G.E.C. Type G.M. 4), enclosed in a conventional lead castle. The sensitivity of the counter was frequently checked with a uranium standard. Counts were recorded on a scaling unit Type 200 together with a power unit Type 200 and a probe unit Type 1014. The samples of the spinal cord were prepared by sectioning transversely selected 5 mm. lengths into ten slices, which were then laid in a shallow depression (14 mm. diameter and 3 mm. depth) in standard aluminium trays. All trays were carefully tested for traces of residual radioactivity before re-use. Comparable slices of medulla oblongata and cerebrum covering about the same superficial area on the trays were also prepared. All specimens were situated 6 mm. from the window of the G.M. tube at the time of counting. This standard procedure enabled the radioactivity of the different portions of the cerebrospinal axis to be determined. At the conclusion of counting the dried specimens were weighed. A sample of the radio-iodinated serum protein-toxin solution was similarly spread and dried on a tray, and its radioactivity determined at a distance of 47 mm. from the tube window. This permitted quantitative comparisons to be made between the tissues obtained in one series of experiments and those in another, although the radioactivities of the inoculated protein solutions differed.

(viii) *Corrections applied to counts on samples.*—(a) *Background:* A large number of background counts were made with this apparatus during the experiments; they showed a mean of 8 counts per minute. Background counts, usually alternating with those on the specimens, were subtracted from the counts obtained with the tissue samples.

(b) *Resolving time:* Since the counting rate with the tissue samples never rose above 500 c.p.m., no correction for this factor was needed.

(c) *Time of counting:* In most cases the counts were made over a period of 4 minutes with the aid of a Ministry of Supply Timing Unit Type 1003, though when they were more than ten times the background value they were often limited to one minute. These short, but adequate, times employed were made necessary by the large number of determinations required and by the relatively short half-life of the isotope used. For records, all counts were expressed as c.p.m.

(d) *Weights of tissue specimens:* Owing to variations in the weights of the different tissue samples, all records were reduced to c.p.m. per 10 mg. dried tissue.

(e) *Self-absorption:* To lessen the variability inseparable from this factor in all beta emission counting, the tissue slices were all cut as closely as possible to the same thickness.

(f) *Isotope decay:* To render all measurements of radioactivity in any single series of animals comparable with one another, individual counts were adjusted on the basis of an estimated half-life of 8·0 days for this isotope.

(g) *Variations in radioactivity of different batches of protein solution used:* Since the different preparations of iodinated serum protein solutions used varied somewhat in their degree of radioactivity (see above), all counts on the different rabbit tissues were re-calculated on the assumption that protein solutions of the same radioactivity strength had been used throughout the whole series of experiments. Thus when the radioactivity of a particular preparation of inoculum was above the midway value, the counts obtained later on the tissue samples were reduced proportionately, and *vice versa*. The factors used for this correction were never large, however, for they ranged between 0·66 and 1·31.

RESULTS.

The Effect of the Intrasciatic Injection of Antitoxin on the Severity of the Intoxication Caused by the Inoculation of Toxin into the Contralateral Sciatic Nerve Trunk.

The following experiments were carried out for two reasons : Firstly, to determine whether there is evidence that proteins such as antitoxins pass centrally along large nerve trunks in the same way as is believed to occur with tetanus toxin. Secondly, to try to lay down an " immunological blockage " within the nervous system which would prove an obstacle to the ascent of the toxin, but to do so in such a way as to preclude the possibility envisaged by Abel *et al.* (1938) of mutual neutralization by contamination such as might happen when, as in comparable experiments made by earlier workers, the toxin and antitoxin are both injected *at nearby sites in the same limb.*

Twelve rabbits all received the same total protecting dose of crude horse serum antitoxin : in six, which served as controls, the whole dose of 0·04 ml. was given intravenously ; in the other six the dose was divided, 0·004 ml. being given intravenously to neutralize any toxin that might gain access to the circulation, and the remaining 0·036 ml. directly into the right main sciatic nerve trunk. Six hours later all twelve rabbits were inoculated with 0·04 ml. of toxin solution containing 0·5 mg. of the dried toxin into their left main sciatic nerve at about the same anatomical level. The animals were operated on in successive groups of four, those that received intravenous injections only being alternated in each group with those that were given combined intrasciatic and intravenous injections of antitoxin. The rabbits were closely observed for the ensuing 100 hours, and the times of any deaths recorded. No deaths except those caused by the specific intoxication were observed. The results are set out in Table I.

TABLE I.—*Survival Times of Rabbits given only Intravenous and of those given Combined Intrasciatic and Intravenous Tetanus Antitoxin, after Injection of Tetanus Toxin into the Contralateral Sciatic Nerve Trunk.*

Intravenous only.		Combined intrasciatic and intravenous.	
Rabbit number.	Survival time (in hours).	Rabbit number.	Survival time (in hours).
D24	21	D23	>100
D26	16	D25	40
D30	22	D29	>100
D32	>100	D31	91
D37	60	D36	69
D39	18	D38	>100

These experiments show that the rabbits which had received most of their antitoxin into the contralateral sciatic nerve trunk lived significantly longer (by Fisher's " t test " $P = <0{\cdot}05$) than those to which it was given only by the intravenous route. Since the latter control group particularly had ample antitoxin in their blood to neutralize any toxin that might escape into the circulation, it seems reasonable to attribute this longer survival to a centripetal movement of some of the antitoxin along this large nerve trunk, and thus to its subsequent

neutralization within the substance of the spinal cord of the toxin which had similarly ascended along the opposite sciatic nerve trunk.

The Effect of Sclerosis of the Sciatic Nerve Trunk on the Severity of the Intoxication Caused by Subsequent Intraneural Inoculation of Toxin at a more Distal Site.

The purpose of the following experiments was to find out whether the creation of a zone of sclerosis in a main nerve trunk which was unaccompanied by paralysis of the muscles that it innervates, affects the severity of the intoxication produced when toxin is injected into the same nerve trunk at a more distal site some weeks later.

The intraneural injection of the sclerosing solutions, ethanol oleate and quinine-urethane, produced during the following three weeks marked reactive changes in the connective tissues of the nerve trunk. We are indebted to our colleague, Dr. G. A. Thomas, for the following notes on the histological features of the affected portion of the nerve trunk :

" The connective-tissue reaction is maximal in the epineurium of the sclerosed nerve, which has increased considerably owing to the laying down of large numbers of reticulin and collagen fibres. The perineurium of the sclerosed nerve shows less reaction, although almost twice as thick as in the control nerve. This increase in thickness seems mainly due to an increase in the amount of collagen present. Proliferation of cells in the endoneurium has increased the number of nuclei present in a transverse section of the sclerosed nerve by about four times.

" The great majority of the nerve fibres of the sclerosed nerve are intact, but isolated fibres show signs of degeneration, possibly due to mechanical injury by the hypodermic needle.

" The number of blood-vessels present within the sclerosed nerve is greater than in the control nerve. In one branch (the nervus gastrocnemius medialis) the number of blood-vessels has increased about four times, and the endothelial and smooth muscle nuclei associated with the vessels show a corresponding increase compared with the normal control nerve. The various nuclear populations of the control nerve correspond very well with the figures obtained by Thomas (1948) for a series of such nerves.

" The size of the sclerosed nerve, excluding the epi- and perineurium, has increased a little compared with the control nerve. This increase is probably due to oedema between the nerve fibres."

Six rabbits whose main sciatic nerve trunks had been injected with sclerosing solution about three weeks previously were each given 0·04 ml. of crude horse serum antitoxin intravenously ; six hours later they all received an intrasciatic inoculation of 0·04 ml. of toxin solution containing 0·5 mg. of the dried toxin at a site several cm. distal to the sclerosed portion of the trunk. At the time these inoculations were made, four of the rabbits showed no sign of any muscular disability of the leg on the sclerosed side, two showed a slight weakness which became apparent on movement. The survival times of these animals after inoculation of the toxin are shown in Table II. The rabbits used as controls in the antitoxin experiments described above were used for the same purpose for this group also, since, except for the prior sclerosis of the nerve trunk, they were treated in exactly the same way.

From Table II it can be seen that the sclerosis of the nerve trunk above the

TABLE II.—*Survival Times of Control Rabbits and of Rabbits whose Sciatic Nerve Trunks had undergone Sclerosis, after Intrasciatic Inoculation with Tetanus Toxin.*

Controls.		Sclerosed.	
Rabbit number.	Survival time (in hours).	Rabbit number.	Survival time (in hours.)
D24	21	D17	>100
D26	16	D18	>100
D30	22	D27	>100
D32	>100	D28	>100
D37	60	D34	>100
D39	18	D35	>100

point of subsequent inoculation of the toxin greatly mitigated the course of the intoxication.

The Centripetal Movement of Proteins in the Sciatic Nerve and Spinal Cord.

Tetanus toxin, after distal intramuscular or intraneural inoculation, can often be identified and to some extent titrated in portions of proximal nerve trunks by injecting extracts made from them intraperitoneally into mice. The toxin has seldom, if ever, been recovered from the central nervous system of intoxicated animals, with some component of which, as was first pointed out by Wassermann and Takaki, it seemed to combine irreversibly. Since there is no method other than by its biological activity by which to identify this toxin, we have fallen back on a study of the behaviour under comparable conditions of a protein of similar size and ready recognizability. Experiments designed to disclose any possible centripetal movement of such substances within the cerebrospinal axis were therefore carried out, in which the tetanus toxin was mixed with homologous serum proteins labelled with radioactive iodine before it was injected into the sciatic nerve trunk at the usual mid-thigh level. On the subsequent death of the animal, the distribution of the radioactive iodine at various sites in the cerebrospinal axis was determined with a G.M. counter that was sensitive to beta radiation. The counts obtained at various levels in the nervous systems of eleven rabbits injected with this mixture of toxin and serum proteins were corrected for background, isotope decay, radioactivity of the particular protein prepartion used and weight of tissue examined. The corrected values for the various specimens taken are given in Table III, and set out diagrammatically in Fig. 1.

The interpretation of these experiments will be discussed in detail below ; it will only be necessary here, therefore, to draw attention to the four main factual findings : (i) The counts were highest in the samples from the lumbar cord, and fell progressively at higher levels of the cerebrospinal axis. (ii) The counts recorded tended to be greater at all levels of the cerebrospinal axis in those animals that were found dead 16 hours after the injection of the toxin than in those that outlived this period and were killed after 18 or 24 hours. Both these findings are clearly shown in Fig. 1. (iii) Samples of skeletal muscle removed from the posterior vertebral group in several of the rabbits showed negligible radioactivity. (iv) From the radioactivity of the proximal portions of the right sciatic nerve it is evident that a small centrifugal movement of the protein has taken place into the opposite uninjected nerve trunk.

TABLE III.—*Corrected Geiger-Muller Counts (per min. per 10 mg. Dried Tissue) of Various Segments of the Cerebrospinal Axis, of Dorsal Muscle and Right Sciatic Nerve of Rabbits following Injection of a Mixture of Tetanus Toxin and Radio-iodinated Homologous Serum Proteins into the Sciatic Nerve.*

Rabbit number.	Hours after injection.	Severity of tetanus.	Spinal cord (cm. from caudal end).									Medulla.	Cerebrum.	Dorsal muscle.	Rt. sciatic (per specimen) cm. from spinal cord.		
			2	5	7·5	10	12·5	15	17·5	20	22·5				2	3	4
D66	16	Found dead	39·8	5·8	8·7	4·5	3·7	—	1·0	2·4	2·8	4·4	*nil*	—	7·5	2·1	*nil*
D67	16	„	67·3	55·3	59·5	60·2	23·4	22·9	18·6	12·7	—	8·0	6·7	—	4·8	*nil*	„
D69	16	„	119·8	110·0	101·5	70·0	21·3	35·5	25·9	14·7	29·3	22·3	11·3	—	12·1	7·2	3·2
D72	16	„	34·6	47·8	34·8	79·2	36·5	26·1	27·7	27·3	18·6	21·9	32·8	1·9	9·4	*nil*	*nil*
D73	16	„	47·4	26·1	41·1	16·9	39·8	36·6	31·8	37·0	24·3	30·2	9·5	—	2·8	„	6·1
D76	16	„	17·2	2·7	3·6	1·2	2·2	1·5	*nil*	0·3	0·8	2·0	0·3	0·5	10·5	1·2	1·4
D77	16	„	16·1	18·8	7·2	11·1	10·3	10·3	5·8	4·1	4·1	4·6	2·7	—	1·5	*nil*	*nil*
	Averages		48·9	38·1	36·6	34·7	19·6	22·2	15·8	14·1	13·3	13·4	9·0	—	6·9	1·3	1·3
			100	78	75	71	40	45	32	29	27	27	18				
D71	18	Generalized *in extremis*	44·9	31·7	21·5	14·6	18·0	14·5	12·5	10·1	14·4	21·6	*nil*	*nil*	15·2	1·9	2·3
D68	24	Generalized	15·2	10·2	4·7	7·3	9·8	6·0	8·3	6·4	5·4	4·6	„	—	7·8	2·4	3·2
D70	18	Hindquarters	41·5	7·3	1·4	2·3	3·5	2·4	10·6	2·9	4·8	4·9	0·2	*nil*	6·6	1·9	*nil*
D75	24	„	23·9	5·2	5·3	3·3	2·7	3·9	4·1	3·8	2·4	4·0	0·5	1·2	2·6	1·8	„
	Averages			31·4	13·6	8·2	6·9	8·5	6·7	8·9	5·8	6·8	8·8	*nil*	8·1	2·0	1·3
				100	43	26	22	27	21	28	19	22	28	„			

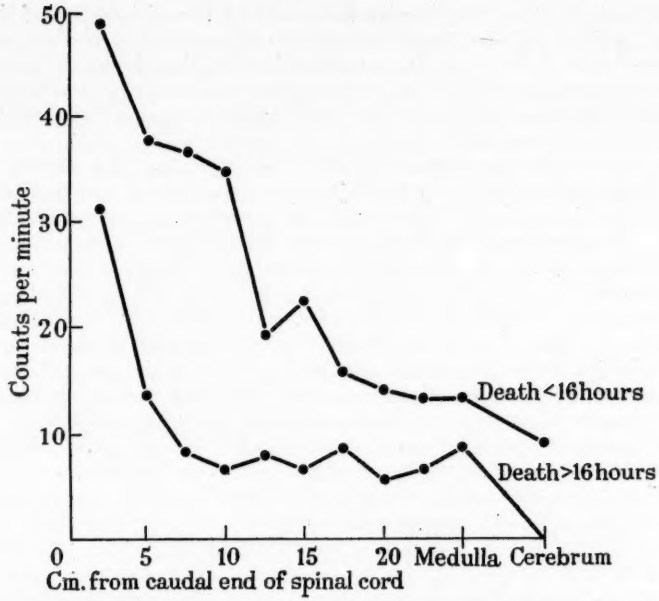

FIG. 1.—Corrected Geiger-Muller counts (per minute per 10 mg. dried tissue) of samples taken from various levels of the cerebrospinal axis after an injection of a mixture of tetanus toxin and radio-iodinated homologous serum proteins into the left sciatic nerve.

DISCUSSION.

The centripetal movement of tetanus toxin in nerve trunks.

Our experiments on antitoxin blockage and nerve sclerosis resemble in their essential features some of those described by earlier investigators, but their planning has been modified in certain respects to meet criticisms directed by Abel and his colleagues against this former work.

Prominent amongst the evidence brought by Meyer and Ransom (1903) in favour of the centripetal movement of tetanus toxin along motor nerve trunks was their finding that after the injection of antitoxin into the main trunk of the sciatic nerve, the distal subcutaneous injection of toxin into the same leg failed to elicit typical local tetanus. They believed that the antitoxin present in the nerve trunk neutralized the ascending toxin *in situ*, and thus prevented its access to vulnerable structures in the spinal cord. These experiments were repeated with similar results by Teale and Embleton (1919–20), who likewise concluded that " the injection of tetanus antitoxin into the sciatic nerve prevents the occurrence of tetanus in that limb, or at least greatly delays the onset and diminishes the severity of the symptoms." The validity of these conclusions has been disputed by Abel, Firor and Chalian (1938). They base their main criticism on the technical ground that it is virtually impossible to effect any intraneural injection, either of toxin or antitoxin, without some contamination of surrounding structures

with fluid that returns along the track opened by the withdrawal of the needle. In those experiments in which, after injection of antitoxin into the nerve trunk, no tetanus followed the distal inoculation of toxin, they believed that traces of antitoxin had escaped through the needle puncture and had neutralized the toxin in the extra-neural tissue spaces before it had had time to exert what they believed to be its primary direct action on the nearby muscles.

In our experiments the possibility of the intermingling of antitoxin and toxin through leakage is precluded by the injection of the former into the sciatic nerve of one leg, and the latter at a symmetrically placed site in the nerve of the other leg. In such experiments technical errors due to local neutralization cannot arise. The less severe intoxication in the group of rabbits that had received antitoxin intraneurally than in the control group to which it had been given only intravenously—which is shown by the comparative survival times given in Table I—cannot therefore be attributed to any inadvertent extra-neural combination of the kind contemplated by Abel and his colleagues. Neither can it be presumed to result from the neutralization of the toxin by antitoxin carried by the circulation from the opposite sciatic nerve, for throughout the period of intoxication there is more antitoxin in the blood of the control rabbits than in the blood of those that received most of their antitoxin intraneurally. The best explanation of these findings would seem to be that an antitoxin blockage similar to that of Meyer and Ransom had been laid down, the only difference being that it was in the lumbar cord and not in the sciatic nerve of the same side. Both toxin and antitoxin, ascending simultaneously along opposite nerve trunks, undergo mutual neutralization when they meet in the tissue fluids of the spinal cord.

The first attempt to prevent the onset of local tetanus by causing sclerosis of the regional nerve trunk at a site proximal to that of inoculation of the toxin was made by Teale and Embleton. They injected the sciatic nerve in rabbits with tincture of iodine, egg albumin and horse serum, in the hope of thereby creating a mechanical obstacle which might prevent and would at least delay the ascent of the toxin should it mount to the spinal cord by this route. In their animals this procedure proved successful and the onset of tetanus was postponed or averted. These experiments were later repeated by Abel, Firor and Chalian, who placed a wholly different interpretation on their results. They found " that the injection of 0·05 c.c. of tincture of iodine or of 90 per cent alcohol into the sciatic of the rabbit or dog injures the nerve to such a degree that complete paralysis of the lower leg promptly ensues. The *intraneural injection of such substances as tincture of iodine is the equivalent of nerve section* for the time being ; muscles paralysed by either method cannot be stiffened by the injection of toxin." The above statement, which they have emphasized by italics, is completely at variance with an equally categorical one made by Teale and Embleton.

The introduction of improved sclerosing fluids for various surgical purposes has enabled us to repeat these experiments under more favourable conditions. Of the six rabbits whose sciatic nerves were injected with ethanolamine oleate or quinine-urethane solutions, four showed no sign of paralysis, one slight paresis, and one, which had injured its foot slightly, tended to drag its leg though still capable of active movement. None of them was therefore incapable of responding to the distal inoculation of toxin by local spasticity because of any interruption of the lower motor neurones such as was apparently brought about by Abel and

his colleagues' more irritant agents. The fact that local tetanus was absent or delayed with such sclerosis must consequently be attributed to some interference with the ascent of the toxin to the lumbar cord.

In addition to their support for the belief that the motor nerve trunks form the main afferent pathway for the toxin to ascend to the spinal cord, these experiments throw some light on which component of the nerve is concerned in the process. Since the time of Marie and Morax (1902) and Meyer and Ransom, many investigators have regarded the axones as the conductors of the toxin, in spite of the absence of any evidence for fluid movements in the axoplasm of normal fibres. The finding in the present experiments that the axones remain functional below the sclerosed site, yet the centripetal movement of the toxin is impeded, is difficult to reconcile with the theory of an intraneuronal pathway for the toxin. If this is rejected, however, two possible intraneural routes still remain: the true lymphatics which lie in the epineurium and the tissue spaces (known by German histologists as "lymphräume" in distinction to lymphatics) that lie between the individual fibres. Spread by lymphatics can now be excluded, for modern work has shown that these vessels drain centrally into lymph nodes in the prevertebral tissues, and not into the thecal space as was formerly believed (see Abel *et al.*, 1935*a*, for full discussion). Numerous observers (Gumprecht, 1895; Robertson, 1916; Teale and Embleton) have believed that the tissue spaces that lie between the individual fibres provide the necessary channels. Among tissue spaces these clefts are unique, for they possess a linearly orientated arrangement, and extend with little change of calibre or internal obstruction all the way from the peripheral muscles to the nuroglia of the cord. Their opening up is readily accomplished under light pressure, and experiments made with bacteria (Homen and Laitenen, 1899; Orr and Rows, 1913–14; Teale and Embleton) and with indian ink (Brierley and Field, 1949) show how readily even small particles can pass along them. When the nerve trunk has undergone sclerosis as a result of an injected irritant, the proliferation of its connective tissues impairs its ability to act as a conducting pathway for tissue fluids.

The ascent of tetanus toxin and other proteins along the spinal cord.

The most convincing support for the belief that tetanus toxin once inside the lumbar cord becomes transported in a cranial direction has come from experiments in which, before the injection of the toxin into the hind limb, the spinal cord has been transected above the lumbar enlargement. Meyer and Ransom gave two kittens, one of which had had its spinal cord divided between the second and third lumbar segments shortly before, an intrasciatic injection of toxin followed at once by a large subcutaneous dose of antitoxin. The animal with the intact cord died with typical ascending tetanus in less than five days, while the other, after becoming spastic in its hind quarters, showed no signs of generalized tetanus and remained active for the following three weeks. Experiments of the same kind, and with the same general results, have been made on dogs by Tiberti (1905) and Firor, Lamont and Shumacker (1940), and on monkeys by Friedemann, Hollander and Tarlov. Permin (1914) tried to obstruct the ascent of the toxin within the cord by a procedure which entailed less trauma, and permitted the unlimited survival of the protected animals. He found, like others, that when toxin was injected intramuscularly in the thighs of rabbits and was followed by

antitoxin intravenously, typical fatal ascending tetanus developed. But if antitoxin was injected into the lumbar cord, the animals subsequently showed neither local nor ascending tetanus.

In view of the well-known difficulty of recovering ordinary lethal doses of tetanus toxin from mixtures with brain and spinal cord, probably because of its irreversible combination with some protein specific to these tissues (Marie and Tiffeneau, 1908), we have approached the problem of a possible migration of the toxin in the cerebrospinal axis in a less direct manner. Brierley and Field recently recorded their important finding that radioactive phosphorus, injected in the form of sodium phosphate solution into the sciatic nerve trunks of rabbits, could sometimes be identified two to four hours later high in the spinal cord and even occasionally in the brain stem. The readiness with which such a diffusible substance is lost by escape into blood capillaries is a handicap for the study of tissue fluid movements. We have repeated these experiments, employing, to avoid immunological complications, the rabbit's own serum proteins, which we have made radioactive by causing them to take up small quantities of I^{131}. This introduction of iodine into the tyrosine of such proteins, unless carried to a much higher degree than we have used, causes no serious changes in their immunological properties (Kleczkowski, 1940 ; Haurowitz *et al.*, 1941 ; Wormall, 1950 ; Banks *et al.*, 1951). Moreover, once the substitution has been effected, the iodine remains firmly attached to the tyrosine (Eisen and Keston, 1949), and consequently, so long as the latter remains a constituent of the protein, to the serum protein molecules themselves. In experiments such as the present ones, labelled proteins have two advantages over sodium phosphate ; firstly, being much larger molecules they are retained longer in the tissue spaces, and therefore register better any drifting movements of the tissue fluids ; and secondly, in point of molecular size they are nearer to tetanus toxin, which has recently been isolated as, or in close association with, a crystalline protein by Pillemer and his associates (1948).

When careful injections of a mixture of tetanus toxin and radioactive serum proteins are made into the sciatic nerve in rabbits, evidence of radioactivity can usually be found 18 to 24 hours later at all levels of the spinal cord, and sometimes in the brain itself. Since the inoculum used is very small (0·04 ml.) in comparison with the volume of the central nervous system, or that even of the spinal cord, and is injected about the mid-femoral region, it is manifestly impossible for this movement to have arisen from the mere mechanical act of injection. Indeed, the slight fusiform swelling of the nerve that follows injections of this volume shows that the greater part of the inoculum is forced for only a short distance up its trunk. There are strong reasons against believing that the protein is carried to the central nervous system wholly by the blood stream. That some of the iodinated protein is absorbed into the circulation is shown by the slow rise in the radioactivity of the blood, and it was to lessen the risk of this possible complication on the counts on the nervous tissue samples that the animals were exsanguinated as completely as possible before dissection. Evidence that the radioactivity of the samples of nervous tissue was not attributable to residual blood is to be found in the negligible counts obtained with the specimens of muscle taken from the rabbits at the same time as the spinal cord. Finally, perhaps the most significant evidence in support of the belief that the iodinated proteins have ascended the cord, rather than been transported to it by the blood stream, is the

gradient of radioactivity along the cerebrospinal axis (see Fig. 1); the counts were highest in samples from the lumbar cord, and decreased progressively in those taken from higher levels.

In conclusion, it is of some interest to make an estimate of what counts such as those recorded in these experiments signify when translated into terms of molecular numbers. With the method of recording employed here, the activity of the dried protein-toxin mixture at the time of its injection into the nerve was about 10^6 c.p.m. per mg. Transformed with the aid of Avogadro's Number, together with an estimated average molecular weight for the serum proteins of 100,000, such a figure means that to give one c.p.m., about 6×10^9 protein molecules must be present in the sample tested. Since in several rabbits 10–20 c.p.m. were recorded from samples taken from the upper end of the spinal cord and medulla, very large numbers of protein molecules derived from the sciatic inoculum must have ascended the cerebrospinal axis during the 16 to 24 hours of survival. Pillemer and Wartman (1947) found that their purified crystalline tetanus toxin had a m.l.d. for mice of $1{\cdot}3 \times 10^{-5}$ μg. Assuming that the protein is of average molecular size for such compounds, this means that the lethal dose for a mouse by intravenous injection is some 10^8 molecules. Even allowing for the lesser species susceptibility and greater size of rabbits, the number of radio-active protein molecules present in the medulla in some of the fatally intoxicated animals in the present experiments is not dissimilar in order to estimates based on Pillemer and Wartman's observations.

SUMMARY.

When tetanus toxin is inoculated into the main trunk of the sciatic nerve in rabbits, the severity of the ensuing intoxication is lessened by a symmetrically placed injection of tetanus antitoxin into the nerve trunk of the opposite limb.

The severity of the intoxication that follows the intraneural inoculation of tetanus toxin into the sciatic nerve is materially reduced in rabbits in which a proximal portion of the nerve trunk had been previously sclerosed by an injection of ethanolamine oleate or quinine-urethane solution.

When a mixture of tetanus toxin and radio-iodinated homologous serum proteins is injected into the sciatic nerve trunk in the mid-femoral region, the presence of radioactivity can be detected in samples taken 16 to 24 hours subsequently from all parts of the cerebrospinal axis.

We are glad to record our indebtedness to Professor A. Wormall and his colleagues for their advice on the radio-iodination of proteins, and on the effects of this upon the immunological behaviour of these substances; to Dr. C. L. Oakley for the gift of specimens of tetanus antitoxin; and to Dr. G. A. Thomas for his note upon the changes in nerve structure induced by sclerosis.

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BODILY REACTIONS TO TRAUMA. FURTHER STUDIES ON THE EFFECT OF ISCHAEMIA ON THE CLOTTING FACTOR OF MUSCLE.

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WE recently (Stoner and Green, 1947) confirmed and amplified the observation of Quastel and Racker (1941) that there is increased clot-promoting activity in saline extracts of ischaemic muscle, these extracts behaving as if they contained more thromboplastin than do those from normal muscle. The study of tissue-clotting factors is not without value, since they play an important part in the production of the toxic effects of the intravenous injection of tissue extracts (Green and Stoner, 1947) including placenta (Schneider, 1947). Furthermore, Knisely and his co-workers (Knisely, Eliot and Bloch, 1945; Knisely, Bloch, Brooks and Warner, 1950; Brooks, Dragstedt, Warner and Knisely, 1950) have demonstrated that the blood passing through an area of damaged tissue is physically altered owing to the formation in it of what they term a "sludge." Although the precise nature of this sludge is not clear, it seems very probable that it consists of small clots.

In the previous paper we were concerned with the characterization of the clot-promoting activity of the extracts from normal and ischaemic muscle. It was shown that they contained a thromboplastin, and it was concluded that the greater activity of the ischaemic muscle extracts was possibly due to the mobilization of the tissue stores of thromboplastin. In the present paper we are concerned with the further testing of this conclusion.

METHODS.

The experiments were performed on albino rats, and the methods used for the production of unilateral hind-limb ischaemia, the preparation of the extracts and the one-stage method for the determination of thromboplastic activity have previously been described (Stoner and Green, 1947). Thromboplastic activity was also determined by a two-stage method based on Herbert's method (1940) for the determination of prothrombin. The reaction mixture in this case was—

1·0 ml. 1 : 25 dilution oxalate rabbit plasma (diluent 0·9 per cent. NaCl).
1·0 ml. glyoxaline buffer pH 7·25.
3·0 ml. muscle extract (1 : 2 extract in 0·855 per cent NaCl, 0·05 per cent $CaCl_2$).

This mixture was incubated at 20°, and from time to time 0·1 ml. aliquots were removed, and added to 1·0 ml. portions of 0·5 per cent fibrinogen, and the clotting time (C.T.) determined at 37°. Two figures were obtained from this experiment,

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the "activation time," the time during which the reaction mixture had to be incubated to give the maximum yield of thrombin, and the minimum C.T. of the fibrinogen. From this latter figure, expressed in reciprocal minutes, the thrombin equivalent of the thromboplastin in the muscle extract was calculated from a standardization curve. This curve was obtained by determining the C.T. of the fibrinogen with different concentrations of thrombin (Bovine Thrombin; Parke Davis & Co.), and when the C.T., in reciprocal minutes, was plotted against the thrombin concentration, a straight line was obtained so that the fibrinogen preparation satisfied the requirements of Astrup and Darling (1942). The fibrinogen used was a pure preparation of human fibrinogen obtained from the Lister Institute through the kindness of Dr. M. Mackay.

In some experiments this procedure was modified (Sternberger, 1947) to stabilize the thrombin formed in the first stage of the reaction. The reaction mixture in this case was—

1·0 ml. oxalate rabbit plasma.
1·0 ml. Michaelis (1931) veronal acetate buffer, pH 7·25.
1·0 ml. ethanol.
1·0 ml. muscle extract (1 : 2 extract in 0·855 per cent NaCl, 0·05 per cent $CaCl_2$).

This mixture was incubated at 20°, and 0·2 ml. aliquots were added to 0·1 ml. 0·5 per cent fibrinogen to determine the activation time and the minimum C.T. of the fibrinogen. The thrombin equivalent of the thromboplastin in the muscle extract was calculated as above.

When the *in vitro* experiments of Quastel and Racker (1941) were repeated the hind limb muscle from two rats was pooled, finely minced with scissors, divided into six roughly equal portions and weighed out into Thunberg tubes, where it was covered with twice the volume of Ringer-Locke's solution (pH 7·0–7·4). One litre of the Ringer-Locke's solution contained 0·9 g. NaCl, 0·42 g. KCl, 0·24 g. $CaCl_2$, 0·15 g. $NaHCO_3$ and 1·0 g. glucose. After 2 or more hours' incubation at 37° under either O_2 or N_2 (O_2 free), the medium in 4 of the tubes was filtered off through Whatman No. 1 paper, and the contents of the remaining 2 tubes were ground up with quartz in a mortar before filtering to give the usual type of muscle extract. The thromboplastin concentration in the filtrate and extract was determined by the one-stage method.

RESULTS.

Since certain qualitative differences had been found between the extracts of normal and ischaemic muscle it seemed best to assume that the concentration of thromboplastin was the same in each muscle, but that the greater clot-promoting activity of the latter extract was due to further qualitative differences.

Calcium Concentration.

The thromboplastin in the two extracts might differ qualitatively in their response to Ca^{++}. Ca^{++} is necessary for the action of thromboplastin, and there is an optimum Ca^{++} concentration for the overall clotting reaction (Owren, 1947) on either side of which the C.T. lengthens. If the optimum Ca^{++} level was different for the two extracts a different thromboplastin concentration might be

suggested if they were only examined, as hitherto, at one Ca^{++} level. This possibility was tested, and it was found that the optimum Ca^{++} concentration was the same for both extracts (Fig. 1), the two curves running parallel at different levels of activity.

Thromboplastin Concentration.

Owren (1947) has shown that the action of several types of thromboplastin diminishes with increasing concentration, and we have confirmed this for brain

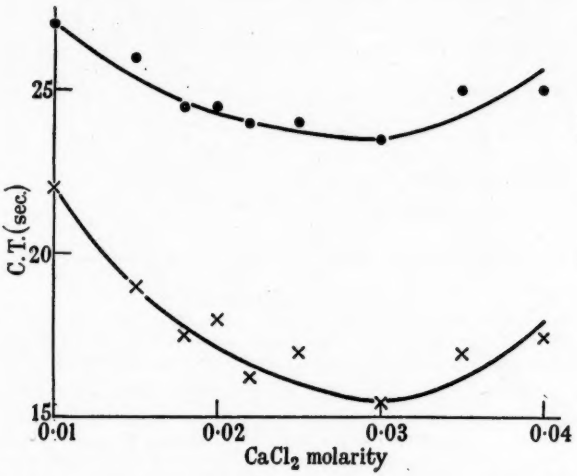

FIG. 1.—The effect of variation in the Ca++ concentration on the coagulation of oxalate rabbit plasma by extracts from normal and ischaemic rat muscle. (Period of ischaemia 5 hr.)

Reaction mixture : 0·1 ml. oxalate rabbit plasma.
0·1 ml. $CaCl_2$ solution.
0·1 ml. 1 : 2 0·9 per cent NaCl extract of muscle.

Temperature 39°.

Control plasma C.T. (0·03 M $CaCl_2$) = 115 sec.

●——● = Normal muscle extract.
×——× = Ischaemic muscle extract.

thromboplastin (Fig. 2). It is possible therefore that, if the extraction of the thromboplastin from the normal muscle was better than from the ischaemic muscle, the two extracts might give the same optimum C.T. at different concentrations. However, normal muscle thromboplastin does not behave, in this respect, like brain thromboplastin, for its activity reaches a maximum when the concentration is quite low and is unaltered by further increases in concentration (Fig. 2). The same is true for the extracts of ischaemic muscle except that the maximum activity was higher. This and the small change in activity over a wide range of concentration shows that the effect is not due to a difference in the extraction of thromboplastin (Stoner and Green, 1947).

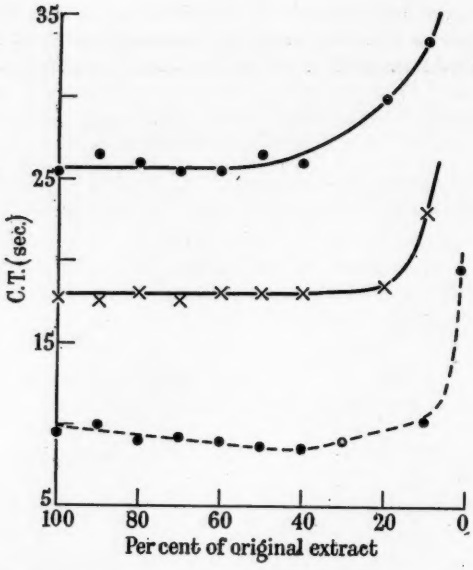

FIG. 2.—The effect of dilution on the clot-promoting activity of extracts of normal and ischaemic muscle and of a preparation of brain thromboplastin. (Period of ischaemia 5 hr.)

Reaction mixture : 0·1 ml. oxalate rabbit plasma.
0·1 ml. 0·03 M $CaCl_2$ (optimum).
0·1 ml. appropriate dilution in 0·9 per cent NaCl of a 1 : 1 0·9 per cent NaCl extract of muscle or of a 4 per cent extract of acetone-dried rat brain prepared with 0·9 per cent NaCl (Owren, 1947).

Temperature 37°.

●——● = Normal muscle extract.
×——× = Ischaemic muscle extract.
● - - - ● = Brain extract.

Additional Clotting Factors.

The experiment illustrated in Fig. 2 shows that neither muscle extract reduced the C.T. of the specimen of rabbit plasma used to a minimal level, since with brain thromboplastin the C.T. was lower than with either. The constancy of the C.T. over a wide range of muscle thromboplastin concentrations suggests that once a certain thromboplastin concentration has been reached it is no longer the limiting factor in determining the C.T. The difference in activity could be explained by the presence of some thromboplastin activator in the ischaemic muscle extract or, conversely, by the absence of an inhibitor normally found in muscle extracts. Recent work by several groups (Quick, 1943 ; Owren, 1944, 1947 ; Nolf, 1945 ; Fantl and Nance, 1946 ; Ware, Guest and Seegers, 1947) on the additional accelerator factors necessary for the first stage of the classical coagulation formula has opened up a possible approach to this problem.

Thromboplastin can be removed from solutions by Seitz filtration. The effect of Seitz filtrates of the muscle extracts on the clot-promoting activity of dilute

TABLE I.—*The Effect of the Seitz Filtrates of Extracts from Normal and Ischaemic Rat Muscle on the Activity of Brain Thromboplastin in the Presence of Optimum Amounts of Ca^{++}.*

Expt. No.	C.T. (sec.) after addition of thromboplastin and Seitz filtrates of: 0·9 per cent NaCl.	Normal muscle extract.	Normal muscle extract (heated).*	Ischaemic muscle extract.	Ischaemic muscle extract (heated).*
1	31·5	30·0	—	23·5	—
2	27.0	22·0	23·0	21·0	23·0
3	27·0	22·0	22·0	21·0	24·0
4	26·0	24·0	—	21·0	—
5	29·0	29·0	—	22·0	29·0
6	29·0	29·0	28·0	24·5	28·0

* Heated at 60° for 30 min.

Reaction mixture : 0·1 ml. oxalate rabbit plasma.
0·1 ml. $CaCl_2$ solution (optimum molarity determined for each experiment).
0·1 ml. Seitz filtrate or 0·9 per cent NaCl.
0·1 ml. brain thromboplastin.

The normal and ischaemic muscle extracts in a particular experiment were prepared from the same rat. Period of ischaemia 4–5 hr.

brain (rat) thromboplastin preparations on rabbit plasma in the presence of optimal amounts of Ca^{++} was observed. It was found (Table I) that such filtrates contained a factor which potentiated the action of the brain thromboplastin. This factor was thermolabile (inactivated by 30 min. at 60°) and, whilst often present in filtrates from normal muscle extracts, was always present in those from ischaemic muscle and in greater amount.

TABLE II.—*The Effect of Seitz Filtrates of Muscle Extracts on the Activity of Muscle Thromboplastin in the Presence of Optimum Amounts of Ca^{++}.*

Experiment No.	C.T. (sec.) after addition of: Normal muscle extract + 0·9 per NaCl.	Normal muscle extract + Seitz filtrate of ischaemic muscle extract.	Ischaemic muscle extract + 0·9 per cent NaCl.
7	27·0	25·5	21·5
8	24·0	23·5	16·5
9	19·0	22·0	17·0
10	26·0	23·5	14·0
11	22·0	23·0	15·0
12	27·0	23·5	14·5
13	30·0	40·0	23·0

Reaction mixture : 0·1 ml. oxalate rabbit plasma.
0·1 ml. $CaCl_2$ solution (optimum molarity determined for each experiment).
0·1 ml. Seitz filtrate or 0·9 per cent NaCl.
0·1 ml. 1 : 2 0·9 per cent NaCl extract of muscle.

Temperature 37°.

The normal and ischaemic muscle extracts in a particular experiment were prepared from the same rat. Period of ischaemia 4–5 hr.

The factor in the Seitz filtrate of the ischaemic muscle extract would usually potentiate the action of the normal muscle extract (Table II), but would not increase its activity to that of the corresponding ischaemic muscle extract.

TABLE III.—*The Effect of Seitz Filtrates of Rat Muscle Extracts on the Coagulation of Recalcified Oxalate Rabbit Plasma.*

Experiment No.	C.T. (sec.).		
	0·9 per cent NaCl.	Seitz filtrate from	
		Normal muscle.	Ischaemic muscle.
1	165	105	54
2	185	190	72
3	185	160	141
4	180	92	125
5	155	113	42
6	155	135	79

Reaction mixture : 0·1 ml. oxalate rabbit plasma.
0·1 ml. 0·02 M $CaCl_2$.
0·1 ml. Seitz filtrate or 0·9 per cent NaCl.
Temperature 37°.

The normal and ischaemic muscle extracts in a particular experiment were prepared from the same rat. Period of ischaemia 4–5 hr.

This more extensive study of the effect of Seitz filtration on the muscle extracts showed that it did not completely inactivate them as previously thought (Stoner and Green, 1947). The persisting activity was very small (Table III) and was usually greater in filtrates from ischaemic muscle extracts. This activity would correspond to very minute amounts of thromboplastin, but it is doubtful whether their action is due to this substance. When the amounts of thromboplastin which might be involved were calculated (Stoner and Green, 1947), it was found that they were too small to account for the potentiation of the brain and muscle thromboplastin shown in Tables I and II. The effect is more likely due to the potentiation of the tiny amounts of thromboplastin occurring in normal plasma by the same factor as described above.

Thrombin Formation.

The greater activity of the ischaemic muscle extracts was not attributed to the presence of pre-formed thrombin (Stoner and Green, 1947), since they only rarely clotted oxalate plasma. Current work supports this view. These extracts did not coagulate pure human fibrinogen when tested either shortly after preparation or after storage overnight at 0°, so that it is unlikely that they contain thrombin at any time. This knowledge was necessary for the next experiment.

A one-stage assay of thromboplastin can only give indirect evidence of its action in converting prothrombin to thrombin. It was hoped that by studying the first phase of the coagulation mechanism in isolation, more direct information would be obtained concerning the greater activity of the ischaemic muscle extracts.

When the two-stage thromboplastin assay was performed with normal muscle extracts it was found that the maximum yield of thrombin and activation time

TABLE IV.—*Conversion of Prothrombin to Thrombin by Normal and Ischaemic Rat Muscle Extracts in the Two-Stage Thromboplastin Assay. Period of Ischaemia = 4 hr.).*

Normal muscle.				Ischaemic muscle.			
Right limb.		Left limb.		Control limb.		Ischaemic limb.	
Activation time. (min.).	Thrombin formed Iowa units per 100 ml. ext.	Activation time. (min.).	Thrombin formed Iowa units per 100 ml. ext.	Activation time (min.).	Thrombin formed Iowa units per 100 ml. ext.	Activation time (min.).	Thrombin formed Iowa units per 100 ml. ext.
24	187	18	200	13	112	8	146
6	240	5	243	5·5	130	5·5	151
10	167	10	163	17	136	8	172
11	167	11	163	5	260	3	312
				†30	440	15	3500
				†18	2100	11	3500

† Second two-stage assay method.

for extracts from the right and left hind limbs of a normal rat were approximately the same (Table IV). Extracts from ischaemic muscle differed in that the thrombin yield was greater and the activation time was shorter (Table IV), so that in both respects these extracts were more active than those from the opposite control limb.

In vitro *Experiments.*

Quastel and Racker (1941) claimed that the medium in which tissues had been incubated anaerobically had a greater clot-promoting activity than that in which they had been incubated aerobically. Our finding (Stoner and Green, 1947) that the thromboplastic activity of muscle extracts was not increased when the muscle was allowed to autolyse *in situ* either aerobically at room temperature or at 37°, or anaerobically at 37°, appeared to be at variance with this. We have since confirmed our initial observations, and have extended the period of autolysis, originally 5 hr., to 24 hr. without observing any increase in thromboplastic activity. To try to explain this apparent difference we have observed the effect of anoxia on the thromboplastin content of muscle *in vitro* in some detail.

When rat muscle was incubated aerobically for 2 hr. as described under "Methods" the thromboplastic activity of the medium was found to be similar in each of the 4 tubes of an individual experiment (Table V, tubes No. 1, 2, 4 and 5). A similar agreement was found between the thromboplastic activities of the extracts prepared by grinding up the contents of the remaining 2 tubes at the end of the experiment to obtain the total amount of thromboplastin present (Table V, Tubes No. 3 and 6). To determine the effect of anoxia Tubes No. 1–3 were incubated aerobically and Tubes No. 4–6 anaerobically, and the results are shown in Table VI, where the thromboplastin concentration of the medium is the mean of the activities in Tubes No. 1 and 2 and Tubes No. 3 and 4 respectively. Under anaerobic conditions the thromboplastic activity of the medium increased whilst the total amount of thromboplastin present remained unaltered. Thus anoxia favoured the diffusion of thromboplastin into the

medium, and this would seem to be a fairly rapid process, since the change appeared to be complete after 2 hr. incubation. These experiments show that the apparent similarity between the *in vitro* and *in vivo* results is not real, and serve to emphasize the difference in the effect of anoxia on muscle thromboplastin activity in the living and dead animal.

TABLE V.—In vitro *Experiments. Thromboplastin Concentration, determined by the One-Stage Assay, in the Medium (Tubes No. 1, 2, 4, 5) after Incubation of Rat Muscle with Twice its Volume of Ringer-Locke's Solution under O_2 for 2 hr. at 37°.*

Experiment No.	14	15	16	17	18
Tube No.	Thrombboplastin concentration (units per ml.).				
1	151	133	45	200	178
2	151	151	32	159	151
3	339	339	295	398	422
4	178*	133	89	178	151
5	133*	159	81	188	188
6	309*	422	309	398	422

* Incubated for 4 hr.

The values for Tubes No. 3 and 6 give the total amount of thromboplastin present. See text for furthur details.

TABLE VI.—In vitro *Experiments. Increase in the Thromboplastin Concentration in the Medium, without Alteration in the Total Amount of Thromboplastin present, on Incubation of Rat Muscle with Twice the Volume of Ringer-Locke's Solution under Anaerobic Conditions.*

Experiment No.	Incubation time (hr.).	Gas phase.	Thromboplastin concentration in medium (units per ml.).	Total thromboplastin (present (units per ml. extract).
19	5	O_2	36	335
		N_2	81	309
20	4	O_2	178	398
		N_2	224	422
21	2	O_2	45	299
		N_2	60	216
22	2	O_2	159	562
		N_2	299	501
23	2	O_2	45	295
		N_2	89	295
24	2	O_2	133	309
		N_2	159	309
25	2	$O_2 + 5\%\ CO_2$	150	500
		$N_2 + 5\% CO_2$	188	500

See text for details.

Effect of Restoration of the Circulation.

Using the one-stage method of assay the maximum increase of 200 per cent in the thromboplastic activity of extracts of ischaemic muscle was found to occur after 4–6 hr. ischaemia. Prolongation of the ischaemia for periods up to 17 hr. showed that this level was maintained during the further anoxia (Stoner and Green, 1947). In the present series of experiments the increase in activity after 4 hr. ischaemia was 175 per cent, the average difference in the activity of extracts prepared from each hind limb of a normal untreated rat being 21 per cent. When the clamp was removed after 4 hr. the activity of the extract from the injured limb rose sharply, being 294 per cent greater than that from the control limb 30 min. later and 528 per cent greater 1 hr. after release. The activity of the injured muscle extracts then began to return to normal in a fluctuating manner, reaching normal levels about 44 hr. after removal of the clamp. Studies were not continued beyond this point. This effect was due to changes in the injured limb, since the activity of the extracts from the contralateral control limb remained within normal limits.

Somewhat similar changes were found using the two-stage assay method (Fig. 3). After removal of the clamp the changes in the activation time and maximum thrombin yield were dissociated, for whilst the thrombin yield began to fall at once, the activation time continued to shorten during the next 2 hr. Forty-four hours after release the thrombin yield from the extract from the damaged limb was approaching that of the control limb, but the activation time for the former extract was still considerably shorter than for the latter.

DISCUSSION.

Although our results do not solve the problem of the greater clot-promoting activity of ischaemic muscle extracts, they do help to eliminate some of the possible explanations considered in the earlier paper. One could then only exclude differences in blood contamination and pH as possible causes. Now, differences in the Ca^{++} content of the extracts and in the degree of extraction of the muscle can also be excluded. Although our results cannot be explained by an easier extractability of thromboplastin from ischaemic muscle, nevertheless the greater diffusibility of thromboplastin from such tissue, seen in the *in vitro* experiments, may play an important part in the production of pathological changes in these tissues.

The greater activity of extracts from ischaemic muscle seems to be due, either to the presence of more thromboplastin or activator/accelerator factors, or to the absence of normally occurring inhibitors. Since the general trend in ischaemic tissue is towards the breakdown of its components the formation of new protein seems unlikely. Yet, thromboplastin may normally exist in the tissues in an inactive form which is changed to an active form during ischaemia. An analogy for this might be the conversion of ovalbumin to plakalbumin by proteolysis (Eeg-Larsen, Linderstrøm-Lang and Ottesen, 1948). Moore and Fox (1950) have also reported changes in the electrophoretic pattern of the proteins in ischaemic muscle which would support the possibility of such an alteration.

The thromboplastin activator found in greater amount in ischaemic muscle extracts must play a part in the effect, but the results suggest that it does not

account for all of it. Little can be said of the nature of this factor. Its filterability and thermolability suggest that it is a low molecular weight protein like Factor V (Owren, 1947). Yet it is not necessarily Factor V, for under our test

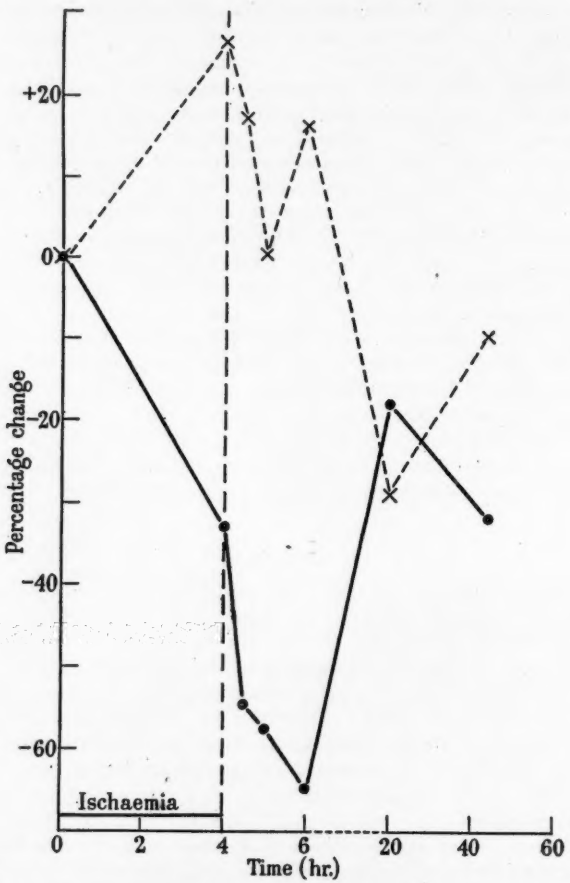

FIG. 3.—Changes in the thrombin yield and activation time recorded in the two-stage assay of extracts from rat muscle during and after a 4-hr. period of unilateral hind limb ischaemia. Results expressed as percentage of values for opposite control limb, each point representing the mean obtained in at least 3 rats.

× - - - × = Thrombin yield.
● —— ● = Activation time.

conditions adequate amounts of Factor V are probably present. It might be a proteolytic enzyme. This possibility has been discussed before (Stoner and Green, 1947), and although it was then found that these extracts would not

digest either casein or fibrin, it cannot be completely excluded, especially in view of such results as those of Travis and Ferguson (1951). In this connection the dissociation of the changes in the activation time and thrombin yield in the two-stage assay of extracts from post-ischaemic muscle is interesting, because the time course of the changes in activation time runs parallel with the degree of proteolysis (Stoner and Green, 1948).

The question of inhibitors has not been specifically studied but a few experiments on the effect of storage on the behaviour of the muscle extracts in the two-stage assay have raised the possibility that the absence of a normal anticoagulant might be concerned in the greater clot-promoting activity of the ischaemic muscle extract. The activity of the normal muscle extract in the two-stage assay increased during the first 24 hours after preparation, after which it remained constant, whilst that of the ischaemic muscle extract appeared to be maximal from the start. The extracts were routinely compared after 24 hours' storage. Attempts to remove anticoagulants of the type described by de Sütö-Nägy (1944*a*) from our extracts with ammonium reineckate (de Sütö-Nägy, 1944*b*) have so far proved unsuccessful.

Much further work is necessary to apportion the credit between these three possibilities, all of which may be involved, and it is difficult to say how much further progress could be made at the moment in view of the technical difficulties and the present disorder in the field of blood coagulation.

SUMMARY.

The differences in the clot-promoting activity of 0·9 per cent NaCl extracts of normal and ischaemic muscle have been investigated further.

Differences in the Ca^{++} requirements and the extractability of the thromboplastin from normal and ischaemic muscle have been excluded as possible explanations of the effect.

The presence of a thromboplastin-potentiating factor in the ischaemic muscle extracts has been demonstrated, and is thought to account, in part, for their greater activity.

Two-stage assay of the thromboplastin showed that with ischaemic muscle extracts the thrombin yield was increased and the time for its formation (activation time) was reduced. After restoration of the circulation to the limb the thrombin yield fell at once, but the activation time continued to shorten for about two hours before commencing to return to normal. The possible significance of this dissociation is discussed. Normal levels were established in the post-ischaemic limb after about 44 hours.

The increase in the thromboplastic activity of muscle extracts during ischaemia could not be demonstrated *in vitro* although, under these conditions, anoxia increased the diffusibility of muscle thromboplastin.

The mobilization of inactive stores of thromboplastin, the presence of potentiating factors and the absence of inhibitors are discussed as possible causes of the effect.

Our thanks are due to the Medical Research Council for defraying the expenses of this investigation, and to Dr. P. A. Owren for his advice.

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FACTORS INFLUENCING THE AGGLUTINABILITY OF RED CELLS. II: THE AGGLUTINATION OF BOVINE RED CELLS PREVIOUSLY CLASSIFIED AS "INAGGLUTINABLE" BY THE BUILDING UP OF AN "ANTI-GLOBULIN: GLOBULIN LATTICE" ON THE SENSITIZED CELLS.

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IN a previous paper (Gleeson-White, Heard, Mynors and Coombs, 1950) we reported that random samples of bovine red cells sensitized with either the Paul-Bunnell antibody or with a rabbit or guinea-pig anti-ox red cell serum differed considerably in their susceptibility to agglutination by appropriate anti-globulin sera. The results obtained from the examination of samples of blood from sixteen oxen were such that those oxen could be divided arbitrarily into three groups on the basis of the susceptibility of their red cells to agglutination by that technique.

Although we used the terms "markedly" and "moderately agglutinable" and "inagglutinable" to describe those three classes of bovine red cell, it should be noted that even the "markedly agglutinable" cells were much less sensitive than sheep red cells to agglutination by the Paul-Bunnell antibody.

Simple absorption experiments showed no obvious difference in the amount of Paul-Bunnell antibody taken up by suspensions of the "markedly agglutinable" and "inagglutinable" class of bovine red cell. Similar results were obtained by Boursnell, Moyle and Coombs (unpublished data), who showed that these samples of bovine red cells sensitized with a guinea-pig anti-ox red cell serum adsorbed equal amounts of a rabbit anti-guinea-pig globulin serum marked with radio-active iodine.

Again, the haemolysin titre of any particular serum used for the sensitization of the bovine red cell suspensions was constant irrespective of the class of cell being tested.

There would appear to be no evidence from these experiments that this variation in susceptibility to agglutination was due to any appreciable difference in the number of antigen receptors present in the three classes of bovine red cell.

We came to the conclusion, therefore, that this phenomenon must be due to some variable character in the structure of the wall of bovine red cells which, although permitting an equal degree of sensitization of all three classes of cell, nevertheless inhibits to a greater or lesser extent the agglutination of these cells.

For instance, the majority of the antigen receptors might be situated at different levels in the cell wall of each class of cell. This would not necessarily prevent the easy access of the sensitizing antibody molecules to the corresponding antigen receptors, or the subsequent union of the former with anti-globulin

* Recipient of a grant from the Agricultural Research Council.

antibody molecules. If, however, these antigen receptors were situated at such a depth that even a sensitizing antibody molecule combined with an anti-globulin antibody molecule was of insufficient " length " for the latter to extend beyond the " outer effective limit of the cell wall," the agglutination of such cells might well be prevented.

If this were the case, it should be possible to obtain the agglutination of suspensions of even the so-called " inagglutinable " class of bovine red cell by repeated alternate treatment with anti-globulin serum and globulin. In other words, by the gradual building up of " anti-globulin : globulin lattices " at the sensitized antigen receptor sites, free globulin receptors would eventually appear at the cell surface. The subsequent addition of further anti-globulin serum should then bring about the agglutination of the cells. This hypothesis is presented in diagrammatic form in Fig. 1(*a*) and (*b*).

To test this hypothesis, suspensions of the three classes of bovine red cell were sensitized with the Paul-Bunnell antibody. These cells were then treated alternately with rabbit anti-human globulin serum and human γ-globulin. By this technique a degree of agglutination equal to the haemolytic titre of the sensitizing antibody was eventually obtained with each class of cell. Furthermore, as might be anticipated from the above hypothesis, the variation in susceptibility to agglutination observed in our previous experiments was reflected in the number of treatments required to obtain a similar degree of agglutination with each of the three classes of cell.

Similar results were also obtained when the cells were sensitized with a rabbit or guinea-pig anti-ox red cell serum and treated in the above manner with the appropriate anti-globulin serum and the corresponding γ-globulin fraction or whole serum.

MATERIALS.

The red cell suspensions and the various sera used in these experiments were prepared in the manner described in our previous paper (Gleeson-White *et al.*, 1950).

Oxen 5153, 5156 and 5157 were again used as the source of " markedly " and " moderately agglutinable " and " inagglutinable " cells respectively.

The human γ-globulin used was a Cohn fraction. This, and all anti-globulin sera and normal human, rabbit and guinea-pig sera employed were first absorbed free of antibodies to bovine red cells.

All sera were heated to 56° for half an hour before use.

METHODS.

Agglutination of Bovine Red Cells, Sensitized with the Paul-Bunnell Antibody, by the Building-up of an "Anti-globulin : Globulin Lattice."

The following is a detailed description of this procedure, which is summarized in Fig. 2.

A two-fold serial dilution of a human serum containing the Paul-Bunnell antibody was made in 0·4 ml. amounts of saline, and similar volumes of a 2 per cent suspension of the bovine red cells to be tested added to each tube. After incubation in a water-bath at 37° for half an hour the tubes were centrifuged

FIG. 1(*a*).— Disposition of antigen receptors beneath the outer effective limit of the cell wall in the three classes of bovine red cells.

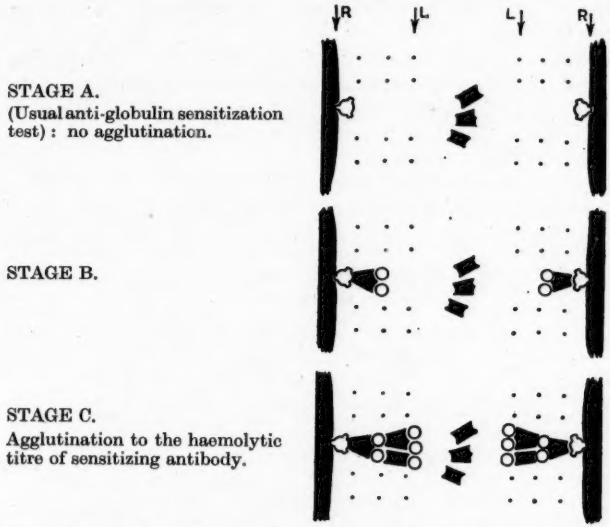
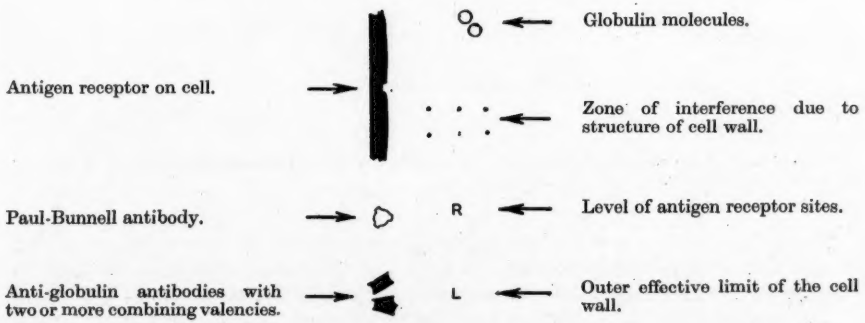


FIG. 1(*b*).—The building up of an " anti-globulin : globulin lattice " on sensitized " inagglutinable " bovine red cells (5157).


Globulin molecules.
Antigen receptor on cell.
Zone of interference due to structure of cell wall.
Paul-Bunnell antibody.
R
Level of antigen receptor sites.
Anti-globulin antibodies with two or more combining valencies.
L
Outer effective limit of the cell wall.

Key to figs. 1(*a*) and 1(*b*).

lightly and the supernatant fluid from each tube discarded. The cell deposits were then washed twice in saline and resuspended in 0·4 ml. amounts of saline.

At this point (Stage A in Fig. 2 and Table I) single drops of the washed cell suspensions were mixed on a tile with equal drops of a 1 in 20 dilution of a rabbit anti-human globulin serum. The tile was then rocked gently by hand at room temperature, and the degree of agglutination read at five-minute intervals over a period of half an hour.

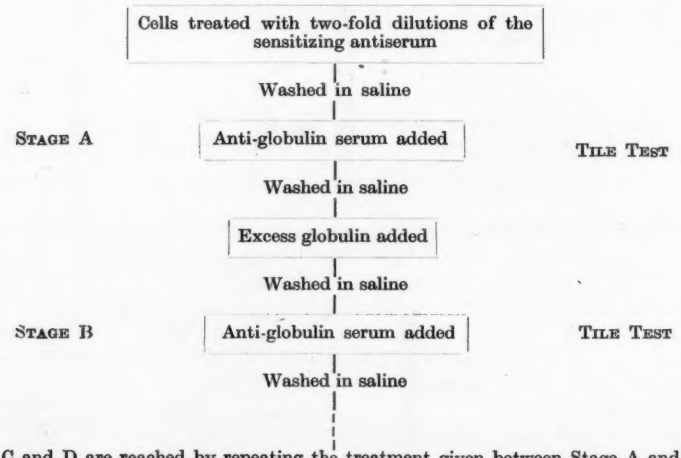
At the same time 0·4 ml. amounts of the same diluted rabbit anti-human globulin serum were added to each tube. After incubation in a water-bath at 37° for twenty minutes the tubes were again centrifuged lightly, the supernatant fluid discarded, and the cell deposits washed twice in saline and re-suspended in 0·4 ml. amounts of saline.

This procedure was then repeated using a 0·14 per cent solution of human γ-globulin instead of the rabbit anti-human globulin serum.

At the completion of this treatment with rabbit anti-human globulin serum and human γ-globulin, another tile test was carried out in the same manner as before (Stage B in Fig. 2 and Table I).

The cell suspensions were then given one or two more treatments with rabbit anti-human globulin serum followed by human γ-globulin, tile tests being carried out at the completion of each treatment (Stages C and D in Fig. 2 and Table I).

FIG. 2.—Summary of the procedure used for the building-up of " anti-globulin : globulin lattices " on sensitized bovine red cells.



Stage C and D are reached by repeating the treatment given between Stage A and B once or twice more respectively.

The only variation in technique as the experiment proceeded was the reduction by 0·05 ml. of the volume of reagents added to the tubes after each tile test to compensate for the volume of cell suspension removed for those tests.

The anti-globulin sensitization tests carried out on tiles at each stage of the experiment were controlled by a duplicate series of tests using a 1 in 20 dilution of normal rabbit serum instead of rabbit anti-human globulin serum.

TABLE I.—*The Agglutination of Suspensions of "Markedly" and "Moderately Agglutinable" and "Inagglutinable" Bovine Red Cells, Sensitized with the Paul-Bunnell Antibody by Repeated Alternate Treatments with a Rabbit Anti-human Globulin Serum and Human γ-globulin.*

Dilutions of sensitizing Paul Bunnell antibody (haemolytic titre (1280)).	Cells 5153 "markedly agglutinable."										Cells 5156 "moderately agglutinable."										Cells 5157 "inagglutinable."										Time of reading on tile.
	1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280	1:2560	1:5120	Saline.	1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.	1:2560.	1:5120.	Saline.	1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.	1:2560.	1:5120.	Saline.	
Direct agglutination by sensitizing Antibody	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 min.
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15 ,,
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage A (Fig. 2)	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 ,,
	++	++	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15 ,,
	++	++	++	++	—	—	—	—	—	—	++	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage B (Fig. 2)	++	++	++	+	—	—	—	—	—	—	++	++	+	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	5 ,,
	++	++	++	++	++	+	w	—	—	—	++	++	++	++	w	—	—	—	—	—	++	++	w	—	—	—	—	—	—	—	15 ,,
	++	++	++	++	++	++	+	w	—	—	++	++	++	++	+	—	—	—	—	—	++	++	++	+	w	—	—	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage C (Fig. 2)											++	++	++	++	+	w	—	—	—	—	++	++	++	++	w	—	—	—	—	—	5 ,,
											++	++	++	++	++	++	+	w	—	—	++	++	++	++	++	+	w	—	—	—	15 ,,
											++	++	++	++	++	++	++	+	w	—	++	++	++	++	++	++	+	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage D (Fig. 2)																					++	++	++	++	++	++	+	—	—	—	5 ,,
																					++	++	++	++	++	++	++	w	—	—	15 ,,
																					++	++	++	++	++	++	++	w	—	—	30 ,,
Controls for the above tests in which normal rabbit serum was used in the tile tests instead of rabbit anti-human globulin serum.																															
Agglutination in normal rabbit serum following treatment to Stage B (Fig. 2)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 min.
	++	++	w	—	—	—	—	—	—	—	w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination in normal rabbit serum following treatment to Stage C (Fig. 2)											—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 ,,
											+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination in normal rabbit serum following treatment to Stage D (Fig. 2)																					—	—	—	—	—	—	—	—	—	—	5 ,,
																					+	—	—	—	—	—	—	—	—	—	30 ,,

Key. ++ = very strong agglutination; + = agglutination; w = weak agglutination; — = no agglutination.

RESULTS.

In the first series of experiments suspensions of the three classes of bovine red cell, sensitized with two-fold dilutions of the Paul-Bunnell antibody, were treated alternately with a rabbit anti-human globulin serum and human γ-globulin. The results of these experiments are given in Table I.

It will be seen that, as far as Stage A, this procedure was similar in every respect to the usual anti-globulin sensitization test. No agglutination of cells 5157 was observed at Stage A, but as the experiment proceeded agglutination began to appear, and by the time Stage C was reached it extended well up to the haemolytic titre of the sensitizing Paul-Bunnell antibody.

As observed in our previous paper (Gleeson-White *et al.*, 1950) the sensitization of cells 5153 was detected by the first treatment with anti-human globulin serum. However, the end-point of agglutination of those cells was greatly enhanced by carrying the experiment to Stage B. These experiments again showed that cells 5156 lie between cells 5153 and 5157 in their susceptibility to agglutination.

Similar experiments to those shown in Table I were also carried out with cells 5153, 5156 and 5157 sensitized with either a rabbit anti-ox red cell serum or a guinea-pig anti-ox red cell serum. For the experiments with the rabbit sensitizing antibody a goat anti-rabbit globulin serum and normal rabbit serum were used instead of a rabbit anti-human globulin serum and human γ-globulin. Similarly, with the guinea-pig sensitizing antibody, a rabbit anti-guinea-pig serum and normal guinea-pig serum were used for the building up of the "anti-globulin : globulin lattices." The results of these experiments were similar to those obtained with the Paul-Bunnell antibody. By carrying the experiment to Stage C or D, cells 5157 were again agglutinated to the haemolytic titre of the serum used for sensitization.

A point of interest observed throughout these experiments was that at each stage of the test the agglutination obtained with the anti-globulin serum was greatly reduced or even inhibited by the subsequent treatment with globulin. This inhibition of agglutination was not unforeseen, since, from our hypothesis, the addition of excess globulin after the re-suspension of the cells would be expected to saturate all free receptors of the anti-globulin antibody molecules, and so reduce the degree of agglutination obtained by the previous treatment with anti-globulin serum.

We were also able to demonstrate that the agglutination obtained in these experiments was not due simply to a non-specific adsorption by the sensitized cells of an immune complex of anti-globulin serum and globulin formed during the course of the test. Cells 5157, sensitized with a *rabbit* anti-ox red cell serum, were given repeated alternate treatments with a rabbit anti-*human globulin* serum and *human globulin*; no agglutination was observed. This showed that the anti-globulin serum must combine specifically with the sensitizing antibody molecules on the cells before agglutination can occur.

DISCUSSION.

Unlike the red cells of most animals, bovine red cells are not agglutinated readily by specific antisera. Even when fully sensitized only certain samples can be agglutinated by anti-globulin sera, and then only to a degree far below

that which might be expected from the haemolytic titre of the sensitizing serum.

However, the results of the experiments reported in this paper show that sensitized bovine red cells can be agglutinated by repeated alternate treatment with anti-globulin serum and the appropriate species of globulin or whole serum. Even the class of bovine red cell previously found by us to be inagglutinable in the usual anti-globulin sensitization test is agglutinated up to or beyond the haemolytic titre of the sensitizing serum by this technique.

The mechanism by which these cells are agglutinated is considered to be, *ex hypothesi*, a building up from the sensitizing antibody molecules of " lattices " composed of alternate layers of anti-globulin antibody and globulin.

It should not be assumed, however, that an " anti-globulin : globulin lattice " is necessarily built up in a simple linear fashion from the sensitizing antibody molecule ; this would only be the case if all components were strictly bi-valent. On the contrary, it is far more likely that multi-valency is the general rule, and that the building up of these lattices takes place in the manner suggested in Fig. 1 (*b*).

From this it might be argued that the agglutination of sensitized bovine red cells by this technique is due solely to an increase in the number of free globulin receptors attached to the cells. If, however, this view were correct, then the application of this technique to any sensitized red cell system should enhance the end-point of agglutination by anti-globulin sera. Nevertheless, no such enhancing effect was observed in preliminary trials with rhesus-positive human red cells sensitized with an incomplete anti-D serum.

In our hypothesis we have suggested that the variation in susceptibility of sensitized bovine red cells to agglutination by anti-globulin sera was due to the antigen receptors being situated at different levels in the cell wall of each class of cell. Whether, in fact, these receptors lie within the actual structure of the cell wall or are situated superficially and masked by an unusually powerful field of repellent forces at the cell surface does not disturb this hypothesis. In either case one can still regard these receptors as lying at varying depths beneath the " outer effective limit of the cell wall," which, although permitting the free access of the specific antibody molecules to the corresponding receptors, renders the cells inagglutinable by ordinary methods.

This conception is supported by the results of these experiments, which show that the " anti-globulin : globulin lattices " have to be built up further on cells 5157 than on cells 5153 before the same degree of agglutination is obtained. Cells 5156 were again shown to occupy an intermediate position between the other two classes of cell.

Furthermore, we have confirmed by repeated tests that the degree of susceptibility to agglutination is a constant property of the red cells of individual oxen. An investigation is now being carried out to discover whether this property of bovine red cells is determined genetically or developed at random.

In due course this " anti-globulin : globulin lattice " technique may also prove to be of value in the investigation of many problems in the field of human blood groups.

In our opinion the results of these experiments with sensitized bovine red cells are also of interest since they, in common with the observations of van Loghem, Kresner, Coombs and Roberts (1950) on the prozone phenomenon

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exhibited by certain anti-globulin sera, provide fresh, though indirect, evidence in support of the theory of the multivalency of antibodies and the lattice hypothesis of agglutination.

Summary.

Sensitized suspensions of the class of bovine red cell previously classified as "inagglutinable" can be made to agglutinate by repeated alternate treatments with anti-globulin serum and globulin. The other two classes of bovine red cell behave in a similar manner, but fewer such treatments are required to obtain the same degree of agglutination.

These results support our hypothesis that the antigen receptors of the three classes of bovine red cell are situated at different levels in the cell wall, and that the building up of "anti-globulin : globulin lattices" from the sensitizing antibody molecules to the "outer effective limit of the cell wall" would bring about the agglutination of these cells.

We wish to express our thanks to Mr. A. E. Harding of the Department of Animal Pathology for the numerous samples of blood used in these experiments, and to Mr. H. P. Hudson, Mr. S. W. Patman and Miss M. H. Redfarn for preparing and photographing the diagrams in this paper.

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PRODUCTION OF FERMENTATIVE VARIANTS BY *SHIGELLA SONNEI* AND OTHER "LATE FERMENTING" ORGANISMS.

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THE delayed fermentation of "sugars" is well recognized in many bacterial groups. In the family *Enterobacteriaceae* especially it is common to find organisms which ferment certain substrates only after prolonged incubation.

Many paracolon-like organisms have biochemical reactions which at first resemble those of the *Salmonella* or *Shigella* group, though they are eventually excluded by fermenting lactose, sucrose or salicin on prolonged incubation. Among pathogens some *Salmonella* strains are slow fermenters of certain substrates. For instance, the time required for dulcitol fermentation is of value in the identification of the biochemical types of *Salm. enteritidis*, while late fermentation of sucrose and lactose by *Sh. sonnei* is well recognized.

In addition to the bacterial species and groups which normally ferment certain substrates only on prolonged incubation, there may occur late-fermenting variants of rapidly fermenting strains. For example, we have recently encountered a strain of *Sh. flexneri* type Z, which when first isolated fermented mannitol only after 4–5 days (Cook, 1951).

These late fermentations present a problem of considerable interest. A certain amount of information is already available. The late-lactose-fermenting *Bacterium coli mutabile* is well known for its ability to produce variants which ferment lactose rapidly and retain this characteristic indefinitely even though grown on a medium without lactose (Massini, 1907; Lewis, 1934).

Sears and Schoolnik (1936) described a strain of *Sh. sonnei* which showed the same kind of variation in lactose, sucrose, raffinose and maltose, though Glynn and Starkey (1939) were unable to breed a pure strain from lactose-fermenting papillae of *Sh. sonnei*.

Most of the work described in this paper has been carried out with a single strain of *Sh. sonnei* isolated from a case of dysentery in 1948, though similar results have been obtained with other late-fermenting strains of Gram-negative intestinal organisms.

Isolation of Fermentative Variants.

The "normal" strain of *Sh. sonnei* was plated out on tryptic heart agar containing 1 per cent lactose or sucrose and 0·0025 per cent neutral red, and the cultures were incubated at 37°. After two or three days a few red papillae began to appear, both on single colonies and on the area of confluent growth. During

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the next day or two the papillae increased rapidly in number and showed great variation in size, the older papillae being the largest and usually fermenting most strongly. By subculturing from the papillae on the same medium, it was possible to obtain pure cultures of variant strains which fermented lactose or sucrose in 24 hours. It was possible to show that in liquid media also, for example lactose or sucrose peptone water, variants were produced during the course of fermentation. In general, it was easier to demonstrate such variants with sucrose than with lactose. By growing a sucrose-fermenting variant in lactose peptone water and plating out on a lactose medium, it was also possible to obtain a double variant of *Sh. sonnei* which fermented both sucrose and lactose within 24 hours.

These rapidly fermenting variants bred true over a period of many months on media not containing the specific substrate. As they were apparently able to transmit the new characteristic indefinitely to their descendants, it seems reasonable to refer to them as mutant cells.

In view of the readiness with which papilla formation and fermentative mutants were obtained from *Sh. sonnei* and the apparent association of the multiplication of these cells with late fermentation, it was decided to examine a number of other late-fermenting organisms.

The cultures were inoculated on heart agar plates containing neutral red, the appropriate sugar and sodium deoxycholate, 1 in 400. (Sodium deoxycholate was found to be of some assistance in detecting papillae by localizing the acid produced and thereby intensifying the colour. It was subsequently found that papillae were also produced on the same medium without deoxycholate.) The plates were incubated at 37° and examined daily. Pure cultures of fermenting variants were obtained by plating out papillae on to the same medium, and their fermentative reactions were tested in peptone water containing the appropriate substrate and indicator. Twelve cultures were so examined, two of which (paracolon 30 and *Bact. coli* MB 539) showed late fermentation of two sugars. The results are shown in Table I. With two exceptions late fermentation of a sugar in liquid media was associated with the production, on agar containing the

TABLE I.—*Late-fermenting Organisms Examined for Rapidly Fermenting Variants.*

Organism.	Substrate.	
Paracolon 3	Salicin	. . . All produced variants.
,, 5	,,	
,, 12	,,	
,, 30	,,	
,, 52	,,	
,, MB 503	,,	
,, 4	Lactose	
,, 40	Sucrose	
Bact. coli MB 539	,,	
,, ,,	Salicin	
Salm. enteritidis var. *essen.*	Dulcitol	
Sh. flexneri Z.	Mannitol	
Paracolon 35	Lactose	No variants detected.
,, 30	Sucrose	

same sugar, of fermenting papillae which bred true on subculture. All these variants produced acid from the appropriate " sugar " within 24 hours, though in a few instances gas production was not detected until the second day.

Detailed Investigations with Sh. sonnei.

These results showed that delayed fermentations were often associated with the production of rapidly fermenting variant cells. To investigate more fully the nature of this association further experiments were performed with *Sh. sonnei*.

The " normal " strain of *Sh. sonnei* was sown into 100 ml. of 1 per cent peptone water and of 1 per cent peptone water with 1 per cent sucrose. The cultures were incubated at 37° and samples from each were removed daily. The pH was determined with appropriate indicators, the turbidity was read with a Hilger biochem absorptiometer and viable counts were done by plating measured volumes of suitable dilutions on the surface of agar plates containing sucrose, and the numbers of colonies of both " normal " cells and fermenting mutants were counted.

The results obtained are summarized in Table II. In the sucrose peptone water the pH remained steady for the first three days, but fell rapidly on the fourth

TABLE II.—*Production of Fermenting Mutants of* Sh. sonnei *in Liquid Media.*

N = Number of viable normal cells in millions/ml.
F = Number of viable fermenting cells in millions/ml.

	Peptone water.			Sucrose peptone water.			Lactose peptone water.		
Day.	pH.	N.	F.	pH.	N.	F.	pH.	N.	F.
1	7·4	49	—	7·1	132	—	7·0	112	—
2	7·4	64	—	7·2	217	—	7·0	191	—
3	7·4	151	—	7·1	445	70	7·0	219	—
4	7·4	231	—	5·4	180	415	7·2	352	—
5	7·3	268	—	5·2	55	458	6·0	593	—
6	7·4	160	—	5·2	76	510	5·0	125	—
7	7·4	166	—	5·6	265	640	5·0	16	—
8	7·2	126	—	5·6	260	650	5·0	17	—

day. On plating out, mutant cells were first detected on the third day. By the following day considerable multiplication of the mutant cells had occurred, and this population level was slightly increased during the next few days. The " normal " cells, on the other hand, showed steady multiplication for the first three days, followed by a sharp drop in numbers on the fourth day. This coincided both with the sudden increase of mutants and with the drop in pH.

Results of similar experiments in lactose peptone water on the other hand were variable. The appearance of mutant cells was more erratic and their detection more difficult. In some experiments (as in the one illustrated in Table II) considerable acid production occurred in lactose peptone water, yet no lactose-fermenting mutants were detected on plating out, though in other experiments a few lactose-fermenting mutants did appear.

In general it seemed that late fermentations were in some way dependent upon the production of mutants. In any given culture the speed of fermentation and the proportion of mutant cells appearing or detected would depend on the equilibrium reached between a number of factors, e.g., the rate of mutation, the relative growth rates of normal and mutant cells, the need for enzymic adaptation by mutant cells, and the possible inhibitory effect of " normal " cells on growth or adaptation of the mutants. Experiments were therefore carried out to investigate these factors.

Proportion of mutant cells.

In an attempt to determine the proportion of mutant cells in a population, the " normal " culture was plated out on an optimal medium to obtain the total viable count and on a minimal medium containing the specific substrate as sole carbon source to obtain the " mutant " count. The optimal medium was tryptic digest heart agar and the minimal medium consisted of $(NH_4)_2HPO_4$ 0·4 per cent, NaCl 0·1 per cent, KH_2PO_4 0·1 per cent, tap water and agar 2 per cent, supplemented with nicotinic acid (0·5 μg. per ml.). In a typical experiment the total viable count was found to be about 200×10^6 per ml., while the " mutant " count increased to about 2000 per ml. after incubating the minimal medium for 5 days, giving about 1 mutant per 10^5 total population. This is necessarily a very rough estimate, since the difficulty commonly met with in this type of work was encountered, viz., that of distinguishing the primary mutants in the inoculum from secondary mutants occurring on the plate. It was not possible to produce a medium on which the " normal " strain completely failed to grow, and it was this growth which, although exceedingly poor, may have given rise to secondary mutants.

An observation of some interest was that this feeble background growth appeared to be able to inhibit the growth of mutant cells. When about 100 lactose- or sucrose-mutant cells were seeded on to inorganic medium containing the appropriate sugar as sole carbon source, colonies appeared in 1 day and reached 2–3 mm. in 3 days. When the same inoculum was seeded on a similar plate with the addition of a massive inoculum of normal cells, the feeble growth of the latter in some way delayed the appearance of the mutant colonies by 2–3 days.

Relative growth rates.

Fig. 1 shows the growth of the " normal " culture and the sucrose and lactose mutants in a peptone medium which allowed only minimal growth unless a suitable carbon source was added. In this medium the normal culture and both mutant cultures grew rapidly when glucose was added. In lactose the lactose mutant and in sucrose the sucrose mutant grew well after a delay of some hours, though not so fast as in glucose. An experiment not illustrated in the figure showed that the lactose mutant in sucrose and the sucrose mutant in lactose grew no better than in the peptone medium. The delay of the mutant cultures in utilizing their specific substrate suggested that some degree of enzymic adaptation was necessary.

Further evidence of this was obtained by experiments in which the sucrose and lactose mutants after subculture in medium with and without the specific substrate were grown in sucrose or lactose peptone water (Fig. 2).

Enzymic adaptation.

The work of Monod (1947) has thrown much light on the phenomenon of enzymic adaptation. Working on the fermentation of lactose by *Bact. coli mutabile* he clearly distinguished the part played by hereditary and environmental factors in determining the response of micro-organisms to new surroundings. The *capacity to respond* adaptively is part of the genetic pattern of each cell and is transmitted indefinitely in subculture, being altered in the direction of loss or gain if a mutation occurs. The adaptive *response* itself is only evoked

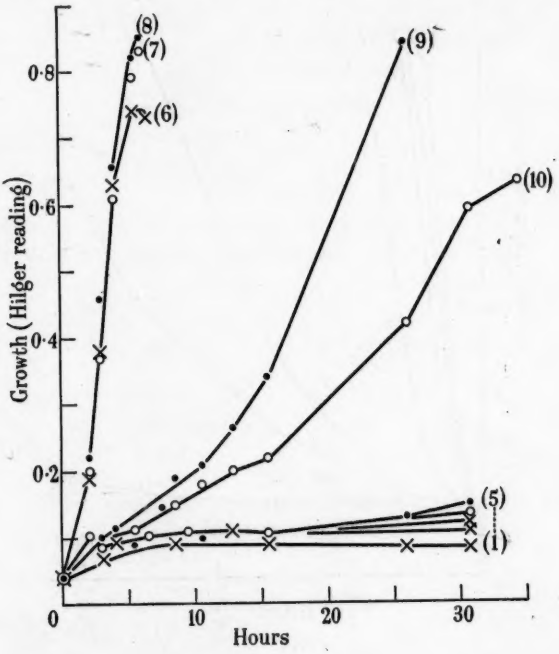

FIG. 1.

as a result of environmental stimuli, and the adapted state is reversible, being rapidly lost in subculture.

Our experiments with *Sh. sonnei* have given results in general agreement with Monod's hypothesis. In order to be able to eliminate the complicating effects of mutation and selection, it was necessary to measure the activity of cells of *Sh. sonnei* in washed suspension. Washed suspensions of cells grown in different ways were incubated with M/20 phosphate buffer and the appropriate carbohydrate (M/50). The rate of fermentation was measured by removing samples at intervals and titrating the acid formed (Hegarty, 1939). In some experiments the oxygen uptake and anaerobic fermentations also were measured in Warburg manometers.

Table III shows the results obtained in a typical acid titration experiment. A washed suspension prepared from a culture of a sucrose-fermenting variant of *Sh. sonnei* grown on nutrient agar showed during 2–3 hours' incubation little or no activity towards sucrose, though it fermented glucose rapidly. A washed suspension, however, prepared from a similar culture grown in sucrose phosphate medium produced acid rapidly from sucrose—at a rate about the same as from glucose. The table also shows that cells subcultured from a sucrose phosphate

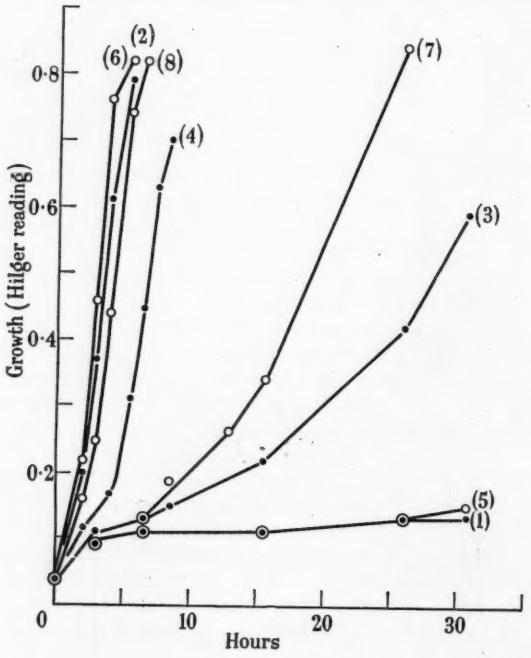

FIG. 2.

medium on to sucrose phosphate agar and then washed off produced acid rapidly from sucrose, but after only one subculture on to agar their sucrose activity dropped to a level near to that of the original agar-grown cells. It is clear that the capacity to utilize sucrose was inherent in the cells and transmitted in each generation, but the activity was manifested only when the cells had been grown in sucrose and presumably adapted to it. Normal cells showed no detectable activity towards sucrose or lactose, though they fermented glucose rapidly.

The lactose-fermenting variant of *Sh. sonnei* on the other hand behaved rather differently towards lactose. Table IV shows the results obtained with washed suspensions of this variant grown in different conditions and, for comparison, with similarly prepared washed suspensions of lactose-fermenting coliform organisms of intermediate type labelled 1433. The coliform organism produced

TABLE III.—*Acid Production* (μ-*equiv./hr./ml.*) *by Washed Cells of the Sucrose Mutant of* Sh. sonnei.

Parent culture grown on—	Experimental culture grown on—	Acid produced by washed cells in presence of— Glucose.	Sucrose.
Agar	Agar	112	9
	Sucrose phosphate agar	112	112
Sucrose phosphate agar	Agar	112	16
	Sucrose phosphate agar	136	120

TABLE IV.—*Acid Production* (μ-*equiv./hr./ml.*) by *Washed Cells of the Lactose Mutant of* Sh. sonnei *and of Coliform Organism* 1433.

Organism.	Culture grown on—	Acid produced by washed cells in presence of— Glucose.	Lactose.
Sh. sonnei	Agar	85	84
	Glucose phosphate agar	120	18
	Lactose phosphate agar	120	120
1433	Agar	220	7
	Glucose phosphate agar	246	<7
	Lactose phosphate agar	246	172

acid from lactose after previous growth on lactose phosphate agar, but not after previous growth on plain agar or glucose phosphate agar, thus behaving in precisely the way described by Monod. Washed cells of the lactose-fermenting variant on the other hand produced acid freely in the presence of lactose even after previous growth in agar not containing lactose. There appears, however, to be some adaptive element involved, since after previous growth on glucose

phosphate agar washed cells of the lactose-fermenting variant of *Sh. sonnei* behaved in the same way as washed cells of organism 1433—that is, they showed only feeble activity towards lactose.

It has been noted earlier that the behaviour of a culture of *Sh. sonnei* growing in lactose peptone water medium was not the same as that of one in sucrose peptone water. In sucrose, multiplication of sucrose-fermenting mutants could be detected before a drop in pH occurred ; in lactose the pH dropped but lactose-fermenting mutants were not readily detected. These differences may be related to differences in basal enzyme content per cell, or differences in speed and extent of adaptation.

DISCUSSION.

The orthodox view that genetically permanent changes in enzymic behaviour cannot be produced in response to environmental stimuli, but only by the selection of spontaneously arising mutants, has not met with universal acceptance. Hinshelwood (1946) believes that bacteria can become permanently adapted to a new environment provided the process of " training " is continued long enough. While our results do not in general support this view, but rather fit the orthodox view, there are certain observations for which we have found no satisfactory explanation. With cultures of *Sh. sonnei* we sometimes found sucrose-fermenting variants which appeared to show differing capacities of fermenting sucrose, and these different strains seemed to breed true. This kind of difference, however, was especially well shown with a late salicin-fermenting paracolon-like organism. The " normal " organism gained no advantage from salicin in a liquid medium in 24 hours ; a " weak " mutant gained no advantage in 6 hours, but grew well in 24 hours ; a " strong " mutant grew very fast in salicin and had nearly reached its maximum population in about 6 hours. We do not know whether these two variants are truly different mutants, and whether it would be possible to select out successively more active mutants, just as mutants of successively greater drug-resistance are selected ; or how much their different behaviour may be due to differing efficiency of adaptation, or to different basal amounts of enzyme.

The production of acid from lactose by *Sh. sonnei* in liquid cultures from which fermenting variants were only occasionally detected requires further investigation. Such variants as were obtained from lactose cultures (in contrast with the sucrose variants) were usually weak fermenters under the aerobic conditions of plate culture. Organisms which produced acid in liquid culture may have been overlooked on the plate.

In general, however, our results show that late fermentations can usually be accounted for by the time taken for variant cells to multiply sufficiently to produce a detectable effect. It is justifiable to call these cells mutants, since they carry in indefinite subculture a new property not present in the cells from which they arose. We cannot at present explain why some of these fermentations are so late, but it is clear that such factors as the rate at which the mutants are produced, the differential growth rate of the " normal " cells and the mutant cells and perhaps an inhibitory effect of " normal " cells on the mutants may all play a part. In addition, our results with sucrose confirm Monod's findings with *Bact. coli mutabile* that the mutant cell is not a cell with a fully developed new enzyme system, but rather a cell endowed with a new capacity for responding adaptively to a specific

stimulus. It is clear that in all work of this kind a distinction must be drawn between the hereditary change which we call a mutation and which seems to be independent of the specific substrate, and the adaptive response to a specific substrate which is not inherited; this demands a combined investigation both into the behaviour of cultures during growth and into the activity of washed suspensions under conditions in which bacterial growth can be excluded.

SUMMARY.

Late fermentation of lactose and sucrose by *Sh. sonnei* was found to be associated with the development of rapidly fermenting variants. These were most easily obtained by subculture from papillae which developed on solid media, though they could also be isolated from liquid media containing the appropriate carbohydrate. The rapidly fermenting variants so obtained bred true when subcultured in the absence of the substrate and are described as "mutants." Similar variants could be isolated from several other members of the family *Entero-bacteriaceae*. With *Sh. sonnei* the main features of the mutants were as follows:

1. The capacity to ferment rapidly during growth was a stable characteristic, unchanged by subculture in the absence of the specific substrate.

2. Washed suspensions of the sucrose mutant rapidly fermented sucrose only when previously adapted by growth on sucrose media; this activity was rapidly lost on subculture in the absence of the substrate.

3. The fermentation of lactose by the lactose mutant seemed to be less dependent on previous adaptation.

The results serve to emphasize the distinction between mutation which is genetically permanent and may occur independently of the specific substrate, and the adapted state which is produced and maintained only in its presence.

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THE VALENCY OF ANTIBODIES.

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THE lattice theory of the precipitation and agglutination of antigens by antibodies is based on the assumption that antibodies are multivalent; unless antibodies have at least two valencies* the large aggregates, which this theory predicates, cannot be built up. The theory and this underlying assumption have been criticized by Hooker and Boyd (1942) and Boyd (1947).

Heidelberger (1939) suggested that "incomplete" antibodies—that is, antibodies that combine specifically with the homologous antigen, but do not, by themselves, form a precipitate with the antigen—have only one valency and cannot therefore build up these aggregates. The matter has become less academic since the importance of incomplete antibodies in relation to erythroblastosis foetalis was discovered.

The bulk of the evidence bearing on the subject has been indirect. It has on the whole favoured the lattice theory, rather than the alternative theory that antigen molecules or particles become coated with antibody molecules that, as a result of their combination with antigen, lose their affinity for water.

However, the lattice theory supposes that antigen and antibody are closely packed in the aggregates in an orderly manner. The combining sites of the antibody molecules must not only be in close contact with the corresponding sites on the antigen molecules, but the molecules must be properly orientated in order that the binding sites may fit. It is difficult to see how the molecules of diphtheria toxin, again properly orientated, can be packed into the aggregate formed by diphtheria antitoxin and rabbit antibody to horse globulin (Smith and Marrack, 1930; Eagle, 1937); or how antigen-antibody floccules, already formed, can take up an amount of complement protein equal to about 72 per cent of the antibody present (Hiedelberger, Rocha e Silva and Mayer, 1941); still more, how the large molecules of ribonucleic acid and its breakdown products can diffuse in and out of ribonuclease-antiribonuclease aggregates so fast that the activity of the enzyme is reduced by only 20 or 30 per cent (Smolens and Sevag, 1942–43).

The most direct evidence is that of Eisen and Karush (1949). They measured the number of molecules of a haptene bound by a molecule of pure antibody at varying haptene concentrations; it was between 1 and 2. By extrapolation to infinite haptene concentration they concluded that the maximum valency

* With regard to the use of the word valency in this connection, we may quote Hooker and Boyd (1942): "Since the reaction appears to be essentially chemical, it is natural to use valence for the number of combining sites or active patches on the surface of reactants. There are many indications that the reactive groups are antigenically complex, i.e., made up of a number of different chemical groups, so the term valence as we use it, although correct etymologically, does not connote quite the same thing as in classical chemistry."

of antibody molecules is 2. It might be supposed that the affinity of protein antigens for antibodies is higher than that of a haptene, and that an antibody molecule could combine with two molecules of antigen at moderate concentrations.

The amounts of antigen combined with antibody in the soluble compounds formed in the presence of a great excess of antigen can be studied by electrophoresis or by ultracentrifugation, if the mobility or sedimentation constant of antigen differs sufficiently from that of antibody. Such studies cannot be made by adding excess of antigen directly to an antiserum, or even to a solution of whole serum globulin, as the great excess of non-specific proteins would hide the antigen-antibody compound. In many cases it is difficult to get a sufficiently pure preparation of anti-body. One method of getting over this difficulty is to form a precipitate of antigen and antibody by adding antigen to serum, to wash his precipitate free from non-specific proteins and to redissolve the precipitate in excess of antigen. This method was used by Tiselius and Kabat (1939), but they did not publish any estimates of the amount of antigen bound by the antibody. Heidelberger and Pedersen (1937) redissolved the precipitate, formed by egg albumin with antiserum, in excess of egg albumin and studied the solution in the ultracentrifuge. From the data given in one experiment (No. 10) we reckon that one molecule of antibody combined with only one molecule of antigen, when 4 times the equivalent amount of antigen was present. Pappenheimer, Lundgren and Williams (1940) studied both, mixtures of excess diphtheria antitoxin with globulin from antitoxic serum, and solutions of toxin-antitoxin precipitates in excess of toxin. They concluded that the valency of diphtheria antitoxin was more than 1 and not more than 2; this is the only direct evidence of a valency for actual antigen that exceeds 1. We have continued studies on these lines. In the earlier experiments we redissolved the precipitates formed by adding antigen to the whole globulin of antiserum in excess of antigen; we assumed that all the precipitated antibody had the mobility of γ-globulin. In later experiments we isolated γ-globulin from antiserum; we (1) added antigen, in approximately equivalent amounts, to the solution of γ-globulin, separated the precipitate and redissolved it in excess antigen, or (2) added the γ-globulin solution directly to excess of antigen. In the last type of experiment we found evidence that when a great excess of antigen was present, more γ-globulin combined with antigen than was carried down in precipitrates formed by antigen and antibody.

METHODS.

Antigens.

The antigens used in the experiments reported were:

1 (*a*). A preparation of crystalline horse serum albumin given us by Dr. E. R. Holiday. This contained 0·2 g. of tryptophane per 100 g. of protein. It was dissolved in 0·9 per cent sodium chloride and filtered through a Seitz filter. No evidence of α_1-globulin (Fig. 1) was found by electrophoresis in barbiturate buffer at pH 8·6 (Fig. 1).

1 (*b*). A solution of the same serum albumin which had not been passed through a Seitz filter. There was evidence of some α_1-globulin in this solution (Fig. 2).

(2) A preparation of crystalline ox serum albumin obtained from Messrs. Armour & Co. There was no evidence of α_1-globulin in solutions of this albumin (Fig. 3). However, Dr. H. Gutfreund tells us that he found that a sample of Armour crystalline ox serum albumin contained some 2 or 3 per cent of a constituent with a sedimentation

constant higher than that of albumin ; and recently, Wallace, Osler and Mayer (1950) reported that a sample contained a trace, estimated at less than 5 per cent, of a component with a sedimentation constant of about 7.

Crystalline egg albumin and crystalline human serum-albumin were also used in experiments which are not reported in detail.

Antibodies.

Rabbits were immunized by Proom's (1943) method and bled 12 to 14 days after an injection of antigen. The sera used in these experiments were obtained by bleeding at the following times after immunization began :

Exp. 3 .	1 and 4 months.	Exp. 8 .	14 months.
,, 6 .	8 months	,, 9 .	5 weeks.
,, 7 .	1 year.	,, 10 .	6 and 7 months.

In the earlier experiments the globulin of the serum was precipitated by half saturation with ammonium sulphate. The precipitate was washed with half-saturated ammonium sulphate, dissolved and dialysed against 0·9 per cent sodium chloride solution to remove ammonium sulphate.

In later experiments γ-globulin was prepared by a modification of Kekwick's (1940) method. Serum was dialysed against 18 per cent sodium sulphate solution, repeatedly changed, at room temperature. The precipitate was centrifuged down or filtered off and washed twice with 18 per cent sodium sulphate. It was re-dissolved and dialysed against 15 per cent sodium sulphate solution, repeatedly changed, at room temperature. The precipitate was centrifuged down and washed twice with 15 per cent sodium sulphate. Precipitation and washing with 15 per cent sodium sulphate were repeated. A solution of the precipitate was cloudy and contained some β-globulin. It was dialysed at 4° C. against a buffer solution (pH approximately 5·4, μ = approx. 0·05) containing 96·5 ml. of M/15 KH_2PO_4 and 3·5 ml. of M/15 Na_2HPO_4 diluted to 135 ml. A precipitate formed which did not dissolve completely in phosphate buffer (pH 8·0, $\mu = 0{\cdot}1$). The supernatant was neutralized with sodium hydroxide and kept at 4° C. overnight ; a slight precipitate formed which was centrifuged off. The supernatant fluid was almost water-clear and contained about three-quarters of the antibody of the original antiserum. In three preparations, S4 (Fig. 4), S6 (Fig. 5) and S7, no component faster than γ-globulin was detected. A third, S8, contained 5 per cent of β-globulin (Fig. 6). This β-globulin was not removed when all the precipitable antibody was precipitated by addition of an equivalent amount of antigen (Fig. 6A) ; none of the precipitable antibody was in the β-globulin fraction. Three other preparations contained some β-globulin ; after further precipitation of two of these by 15 per cent sodium sulphate and dialysis against phosphate buffer (pH 5·4, $\mu = 0{\cdot}05$) preparations S9 and S10, free from detectable β-globulin, were obtained (Fig. 7 and 7A) ; the third (S11, Fig. 19) was used without further treatment.

Precipitation curves.

Progressively increasing amounts of antigen were added to a series of constant amounts of antibody solution. The mixtures were kept at room temperature until floccules formed and then at 4° C. for 2 days. The precipitates were centrifuged down, washed three times with cold saline and the protein estimated by the tyrosine method (Heidelberger and MacPherson, 1943 ; Heidelberger and Anderson, 1944) ; this was checked from time to time by Kjeldahl's method. The supernatant fluids were tested for excess of antibody and antigen.

Precipitation and resolution.

Pilot experiment.—A pilot experiment was made in order to estimate the amount of antibody precipitated with the antigen in the main experiment. To an antibody solution an equivalent or slightly less than equivalent amount of antigen was added. The ratio of antigen to antibody, length of time between mixing and centrifuging and other conditions of precipitation were the same as in the main experiment. The precipitate was centrifuged down, washed twice with cold 0·9 per cent NaCl solution and the protein estimated by Kjeldahl's method. The amount of antibody precipitated was equal to the total protein precipitated minus the antigen protein.

Main experiment.—All apparatus was sterilized by heat or by soaking in 70 per cent alcohol, followed by washing with sterile 0·9 per cent NaCl solution. All stages of the experiment, up to the dialysis before electrophoresis, were done with precautions against contamination. The ratio of antigen to antibody was the same as in the pilot experiment, but larger amounts were used. The precipitate was washed as in the pilot experiment and stirred with a few ml. of 0·9 per cent saline. A concentrated solution of antigen was added in measured lots. The mixture was periodically warmed to 38° C., and usually kept at room temperature during most of the day and at 4° C. during the night. When all or a considerable proportion of the precipitate had dissolved the mixture was dialysed against phosphate buffer pH 8·0, $\mu = 0{\cdot}1$, and examined by electrophoresis.

Direct addition of antibody to excess antigen.

In order to measure the maximum precipitable antibody, the equivalent amount of antigen was added to a sample of γ-globulin solution. After floccules had formed at room temperature the mixture was left for 2 days, with occasional shaking, at 4° C.; the precipitate was centrifuged down, washed three times with cold 0·9 per cent saline solution and estimated by Kjeldahl's method. In the main experiment a measured amount of the γ-globulin solution was added slowly with continual mixing to a large excess of antigen. The mixture was dialysed against phosphate buffer pH 8, $\mu = 0{\cdot}1$ and examined by electrophoresis.

Kjeldahl estimations.

The concentration of protein in the various solutions and in the precipitates was estimated by measuring the nitrogen content by Kjeldahl's method (Hoch and Marrack, 1945).

Electrophoresis.

In the examination of the samples of serum albumin (Fig. 1, 2, 3) the electrophoresis took place in barbiturate buffer of pH 8·6 and ionic strength 0·1. All other electrophoresis experiments were done in phosphate buffer of pH 8·0 and ionic strength 0·1. The patterns were analysed by the method used by Svedberg and Pedersen (1940). The estimates of the individual components were probably accurate to within ±1 per cent of the sum of the components when the peaks were well separated, but often the conditions were less favourable and occasionally the error may have been ±3 per cent. The error in the estimates of the two components of the complex, represented by two peaks in Fig. 9 (Exp. 6), may have been of this order. In this case the two components of the complex, expressed as per cent of the total, might be 11·6 and 18·5 or 17·6 and 12·5 instead of 14·6 and 15·5, the figures given in the account of the experiment.

Calculation of the composition of antigen-antibody complexes.

In experiments in which an antigen-antibody precipitate was re-dissolved the protein in the precipitate was calculated from the result of the pilot experiment; the amount

of antigen added to dissolve the precipitate was known. Hence the total protein was known; it was also measured by Kjeldahl's method in several experiments and the amount found agreed well with that calculated. In experiments in which γ-globulin solution was added directly to excess of antigen, the amount of *precipitable* antibody was known from precipitation curves.

Owing to the great differences between the mobilities of the antigens used and γ-globulin, the antigen-antibody complexes in solution usually showed in the electrophoresis patterns as peaks distinct from the free antigen and from any inert γ-globulin, if present. The ratios of free antigen, complex and inert γ-globulin (if present) to total protein were calculated from the ratios of the areas of the corresponding peaks to the total area of the peaks. As the total amount of protein was known the amounts of free antigen, complex and inert γ-globulin (if present) could be calculated. The combined antigen was the difference between the total antigen and free antigen. The antibody was known from the pilot experiment, and was also calculated as the difference between total complex and combined antigen. As the complex boundary in the descending side was free from excess of the faster-moving antigen, the calculations were based on the proportions of the components found on the ascending side.

The molecular ratio of antigen to antibody was calculated on the assumption that the molecular weights of albumin was 70,000, and that of the antibody 160,000.

RESULTS.

Three experiments other than those reported were done with horse serum albumin as antigen. In these the amount of residual precipitate was too large to be disregarded; as its composition was not known it was not possible to calculate the amount of antibody

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EXPLANATION OF PLATES.

Electrophoresis patterns; pH 8·0 unless otherwise stated; ascending on the left, descending on the right (except in Fig. 13 which has been accidentally reversed).

FIG. 1.—Horse serum albumin, sample 1*a*; pH 8·6.

FIG. 2.—Horse serum albumin, sample 1*b*; pH 8·6.

FIG. 3.—Ox serum albumin; pH 8·6.

FIG. 4.—γ-globulin, S4.

FIG. 5.—γ-globulin, S6.

FIG. 6.—γ-globulin, S8.

FIG. 6A.—γ-globulin, S8, after precipitation of antibody.

FIG. 7.—γ-globulin, S9.

FIG. 7A —γ-globulin, S10.

FIG. 8.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 3.

FIG. 9.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 6.

FIG. 10.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 9A (6.i.50).

FIG. 10A.—Antigen-antibody precipitate dissolved in excess antigen, Experiment 9A (11.i.50).

FIG. 11.—Antigen excess added to γ-globulin, Experiment 7A.

FIG. 12.—Antigen excess added to γ-globulin, Experiment 7B.

FIG. 13.—Antigen excess added to γ-globulin, Experiment 9B.

FIG. 14.—Antigen excess added to γ-globulin, Experiment 10A.

FIG. 15.—Supernatant fluid after precipitation of antibody, Experiment 8A.

FIG. 15A.—Supernatant fluid plus excess antigen, Experiment 8A.

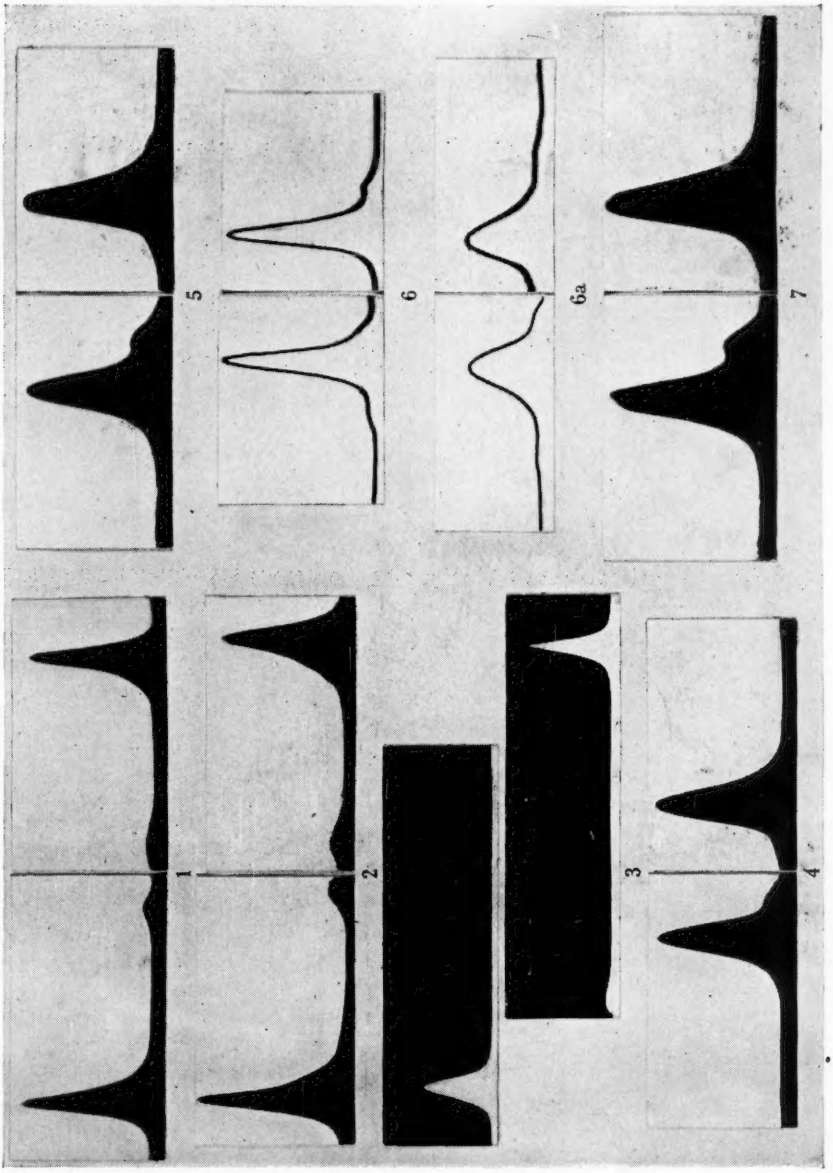
FIG. 16.—Supernatant fluid (Fig. 6A) plus excess antigen, Experiment 9C.

FIG. 17.—Supernatant fluid after precipitation of antibody, Experiment 10B.

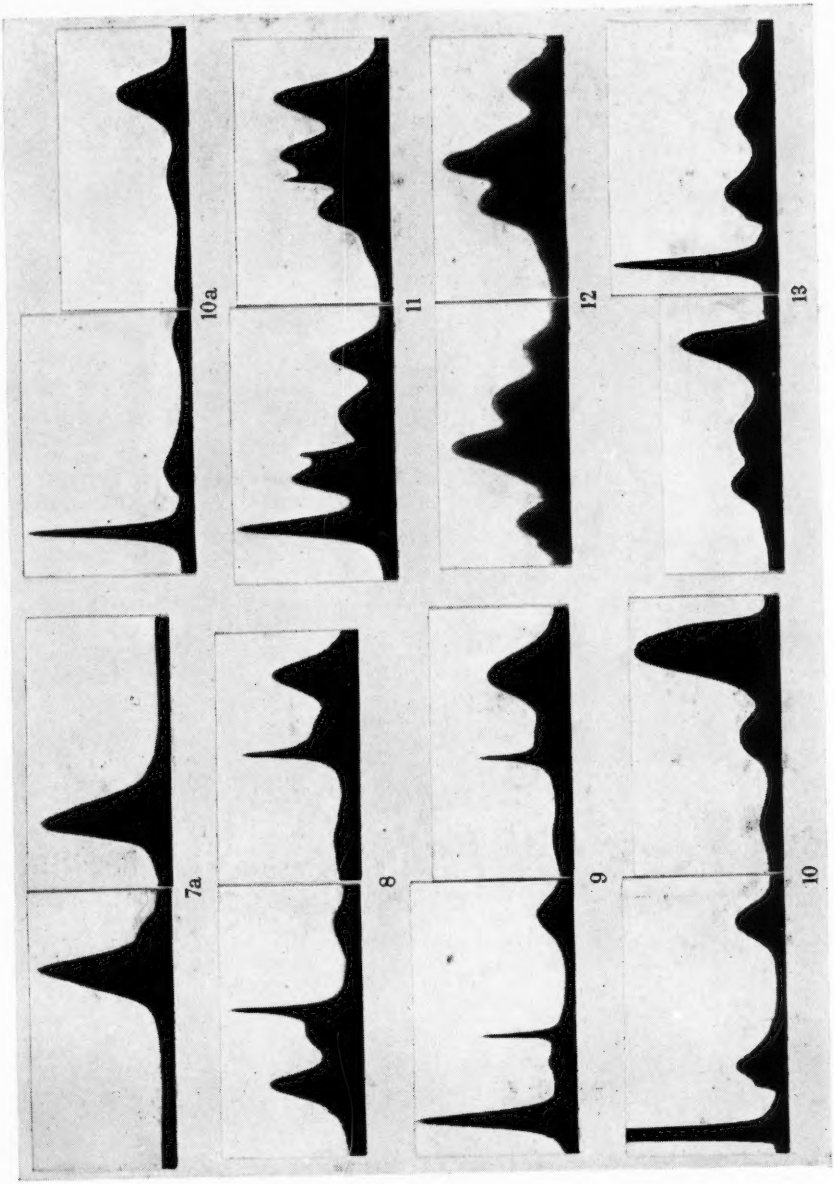
FIG. 18.—Supernatant fluid (Experiment 10B) plus excess antigen.

FIG. 19.—γ-globulin (S11) Experiment 11, ascending.

FIG. 20.—Inert γ-globulin plus ox serum albumin, Experiment 11.



Marrack, Hoch and Johns.



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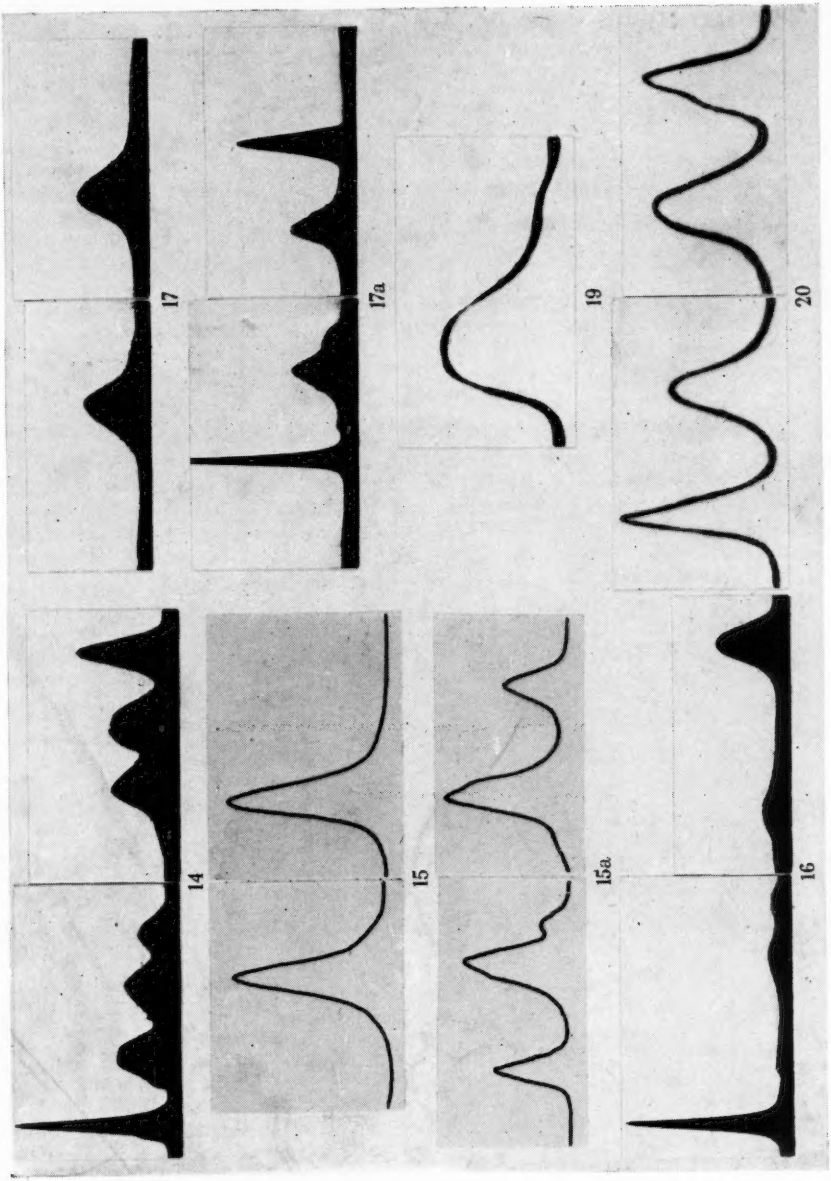


Marrack, Hoch and Johns.

and antigen in the soluble complex. However, in one of these (Experiment 5B) 1·8 mg. of precipitate remained undissolved. Even if this precipitate consisted of antibody only, the molecular ratio would be 1·9; if the precipitate contained two molecules of antibody to one of antigen the molecular ratio would be 1·8.

Experiment 3.

Antigen: Crystalline horse serum albumin (sample 1*a*) (Fig. 1).

Antibody solution: Solution of globulin from antiserum precipitated by half saturation with ammonium sulphate and dialysed against 0·9 per cent NaCl solution; pH adjusted to about 7·5.

Pilot experiment: 2 ml. of antibody solution and 0·8 ml. of 0·039 per cent albumin solution (0·312 mg. of albumin) formed 2·99 mg. of precipitate. Antibody 2·68 mg., or 1·34 mg. per ml.

Main experiment: 12 ml. of antibody solution, 4 ml. of 0·9 per cent NaCl and 0·96 ml. of 0·198 per cent albumin solution (1·90 mg. of albumin).

Antigen added to washed precipitate: 1·2 ml. of 1·89 per cent albumin and a further 0·6 ml. on the following day.

Total albumin added . .	34·0 mg.
Total albumin in mixture .	35·9 mg. (19 times the equivalent amount).

The mixture was kept at 38° C. for a total of 8 hours on consecutive days and at 4° C. during two nights. The bulk of the precipitate had then dissolved and the mixture was dialysed against phosphate buffer, pH 8·0 for 1 day. A small precipitate (0·2 mg.) remained undissolved. Interval between first addition of excess antigen and electrophoresis 3 days.

Original precipitate (calculated from pilot experiment) .	= 17·9 mg.
Albumin added	= 34·0 ,,
Total protein calculated	51·9 ,,
Found in residual precipitate	= 0·2 ,,
Found in supernatant solution	= 51·1 ,,
Total found	51·3 ,,

Composition by electrophoresis (Fig. 8):

	Ascending.	Descending.
Albumin . . .	47·0 per cent .	52·0 per cent.
Complex (1) . . .	19·0 ,, .	18·0 ,,
Complex (2) . . .	34·0 ,, .	30·0 ,,

No component with the mobility of γ-globulin was detected. The complex formed 2 peaks.

Neglecting the precipitate in the calculation:

Total protein in solution	= 51·1 mg.
Total albumin in solution	= 35·9 ,,
Free albumin in solution 51·1 × 0·47 . . .	= 24·0 ,,
Combined albumin	= 11·9 ,,
Complex in solution 51·1 × 0·53	= 27·1 ,,
Antibody in complex, by difference	= 15·2 ,,
Antibody, from pilot experiment	= 16·1 ,,

Ratio of antigen to antibody in complex, $\frac{11·9}{15·2} = 0·78$.

Molecular ratio, 1·8.

Experiment 6.

Antigen: Crystalline horse serum albumin (sample 1*a*) (Fig. 1).

Antibody solution: Solution of globulin precipitated from antiserum by half saturation with ammonium sulphate, dialysed against 0·9 per cent NaCl, neutralized and Seitz filtered.

Pilot experiment: 2 ml. of antibody solution and 0·4 ml. of 0·302 per cent albumin solution (1·208 mg. of albumin) formed 10·3 mg. of precipitate. Antibody 9·1 mg., or 3·03 per ml.

Main experiment: 10 ml. of antibody solution and 2 ml. of 0·302 per cent albumin (6·04 mg. of albumin).

Antigen added to washed precipitate; 6·5 ml. of 2·8 per cent albumin in 3 instalments.

Total albumin added	.	182 mg.
Total albumin present	.	188 ,, (31 times the equivalent amount).

Final volume 8·57 ml., protein concentration 2·73 per cent.

Original precipitate, calculated from pilot experiment	= 51·5 mg.
Albumin added	=182 ,,
Total protein	=233·5 ,,
Protein found 8·57 × 27·3 mg.	=234 ,,

The mixture was kept at room temperature, with repeated shaking, by day and at 4° C. at night, for 9 days, then dialysed, for electrophoresis, against phosphate buffer pH 8. The precipitate was completely dissolved.

Interval between precipitation and electrophoresis, 10 days.

Composition by electrophoresis (Fig. 9):

	Ascending.	Descending.
Albumin . . .	66·7 per cent .	71·4 per cent.
Faster complex . .	14·6 ,, .	10·4 ,,
Slower complex . .	15·5 ,, .	16·0 ,,
Material with mobility between slow complex and γ-globulin . .	3·2 ,, .	2·2 ,,

No component with the mobility of γ-globulin was detected.

Total protein in solution	=234 mg.
Total albumin	=188 ,,
Free albumin (234 × 0·667)	=156 ,,
Combined albumin	= 32 ,,
Total complex (234 × 0·333)	= 78 ,,
Antibody in complex, by difference	= 46 ,,
Antibody, from pilot experiment	= 45·5 ,,

$$\text{Ratio of antigen to antibody, } \frac{32}{45 \cdot 5} = 0 \cdot 70.$$

Molecular ratio, 1·6.

In this experiment the division of the complex into two peaks is clearly seen. The composition of the whole complex agrees fairly closely with that of a mixture in which the two main components had the composition AG_2 and A_2G_3, and the slowest component the composition AG.

Experiment 7.

Antigen: Crystalline horse-serum albumin. This preparation, unlike that used in Experiments 3 and 6, had not been passed through a Seitz filter; it contained a small

amount of α_1-globulin, as shown by asymmetry of the peak in the electrophoresis pattern at pH 8·6 (Fig. 2).

Antibody: S4, solution of γ-globulin (Fig. 4) prepared from antiserum. The precipitable antibody in this solution found by precipitation with an equivalent amount of antigen was 5·15 mg. per ml.; the total globulin was 13·1 mg. per ml.

(A) 5 ml. of S4 (total protein 65·5 mg., precipitable antibody 25·75 mg.) were added slowly with thorough mixing to 1·5 ml. of 4·58 per cent albumin solution (68·7 mg. of albumin, 23 times the equivalent amount) and 0·5 ml. of phosphate buffer pH 8·0. $\mu = 0{\cdot}1$. The mixture was dialysed against phosphate buffer pH 8·0 for 2 days.

Composition by electrophoresis (Fig. 11):

	Ascending.	Descending.
Albumin	34·5 per cent	37·4 per cent
Complex	44·9 „	43·3 „
γ-globulin	20·6 „	19·3 „

Total protein	= 134·2 mg.
Total albumin	= 68·7 „
Free albumin (134·2 × 0·345 mg.)	= 46·4 „
Combined albumin	= 22·3 „
Complex (134·2 × 0·499)	= 60·4 „
Globulin in complex	= 38·1 „
Precipitable antibody	= 25·75 „

Ratio of antigen in complex to *precipitable antibody*, $\frac{22{\cdot}3}{25{\cdot}75} = 0{\cdot}87$.

Molecular ratio, 2·0.

Ratio of antigen in complex to total globulin in complex, $\frac{22{\cdot}3}{38{\cdot}1} = 0{\cdot}586$.

Molecular ratio, 1·34.

(B) After electrophoresis 5 ml. of this solution, containing 45·85 mg. of protein (22·4 globulin, 8·8 precipitable antibody, 23·45 albumin), were taken and 2 ml. of solution S4 (26·2 mg. globulin, 10·3 mg. precipitable antibody) were added, the antigen in the mixture was 11 times the equivalent amount. The mixture was dialysed further and examined after 5 days. 2·25 mg. of precipitate formed which are assumed to have contained 0·4 mg. of albumin and 1·85 mg. of antibody.

Composition of electrophoresis (Fig. 12):

	Ascending.	Descending.
Albumin	15·5 per cent	15·8 per cent
Complexes	53·0 „	54·4 „
γ-globulin	31·5 „	29·8 „

Total protein in solution (72·05 − 2·25)	= 69·8 mg.
Total albumin (23·45 − 0·4 mg.)	= 23·05 „
Free albumin (69·8 × 0·155 mg.)	= 10·8 „
Combined albumin	= 13·25 „
Complex (69·8 × 0·53 mg.)	= 37·0 „
Globulin in complex, by difference	= 23·75 „
Precipitable antibody	= 17·25 „

Ratio of antigen in complex to *precipitable antibody*, $\frac{13{\cdot}25}{17{\cdot}25} = 0{\cdot}77$.

Molecular ratio, 1·76.

Ratio of antigen in complex to total globulin in complex $\frac{13{\cdot}25}{23{\cdot}75} = 0{\cdot}56$.

Molecular ratio, 1·28.

In the first part (A) of this experiment the separation of two complex peaks was conspicuous, the slower peak was steep and narrow. The great difference between the amount of precipitable antibody and the amount of globulin found in the complexes will be discussed later.

Experiment 8.

Antigen: Crystalline horse serum albumin. This preparation had been passed through a Seitz filter, but still contained a little α_1-globulin.

Antibody: S6, solution of γ-globulin prepared from antiserum. The precipitable antibody in this solution found by precipitating with an equivalent amount of antigen was 11·5 mg. per ml.; the total globulin was 45·2 mg. per ml.

1·5 ml. of S6 (total globulin 67·8 mg., precipitable antibody 17·3 mg.) were added slowly with thorough mixing to 5·5 ml. of 0·61 per cent albumin solution (total albumin 33·5 mg. = 17·7 times the equivalent amount). The mixture was opalescent and after it had been kept at 4° C. for 3 days 4·0 mg. of precipitate separated.

Composition by electrophoresis:

	Ascending.	Descending.
Albumin . . .	18·7 per cent .	20·1 per cent.

The complex peaks could not well be separated from the γ-globulin and therefore no estimate was made of the relative proportions of complex and free γ-globulin.

In the calculation it is assumed that the precipitate formed had the composition A_2G and contained 0·7 mg. of albumin and 3·3 mg. of antibody.

Total protein (101·3 − 4·0 mg.)	= 97·3 mg.
Total albumin (33·5 − 0·7 mg.)	= 26·5 „
Free albumin (97·3 × 0·187 mg.)	= 18·2 „
Combined albumin	= 8·3 „
Precipitable antibody (17·3 − 3·3 mg.) . . .	= 14·0 „

Ratio of antigen to *precipitable antibody*, $\frac{8 \cdot 3}{14 \cdot 0} = 0 \cdot 59$.

Molecular ratio, 1·26.

In view of the presence of a large amount of globulin which combined with antigen when a large excess antigen was present in solution, but was not carried down in the precipitate formed with antigen at the equivalence point, a further experiment was made with solution S6.

(A) To 6·5 ml. of a 1·55 per cent solution of S6 (total globulin 100·6 mg., antibody 27 mg.) the equivalent amount of albumin (2·93 mg.) was added in solution in 0·60 ml. The mixture was left at room temperature until it flocculated and kept at 4° C. overnight. The precipitate was then packed by centrifuging. The supernatant was dialysed against phosphate buffer pH 8·0 and examined by electrophoresis; no component with mobility faster than that of γ-globulin was detected (Fig. 15). To this supernatant, after electrophoresis, 0·5 ml. of 4·58 per cent albumin (22·9 mg.) was added; this was 7·8 times the equivalent of the antibody present in the original 6·5 ml. of S6 used: 2 mg. of precipitate formed. No complex was found in the electrophoresis pattern of the supernatant fluid (Fig. 15A); but a slight shoulder appeared on the γ-globulin peak which can be seen if Fig. 15 and 15A are compared.

Experiment 9.

Antigen: Ox serum albumin (Armour); no evidence of α_1-globulin or slower components (Fig. 3).

Antibody: Solution of γ-globulin S8 containing 5·7 per cent of a component with the mobility of β-globulin (Fig. 6); total protein 25 mg. per ml.; precipitable antibody

5·7 mg. per ml. After the precipitable antibody had been removed by an equivalent amount of antigen the faster component made up 8·5 per cent of the total protein (Fig. 6A); this component, therefore, contained no precipitable antibody.

This preparation was examined in the ultracentrifuge by Dr. Kekwick of the Lister Institute and found to be homogeneous.

(A) *Solution of specific precipitate in excess antigen.*

Pilot experiment: 0·9 ml. of S8, 0·9 ml. of 0·9 per cent NaCl and 0·18 ml. of 3·95 per cent albumin solution (0·710 mg.) formed 5·85 mg. of precipitate; antibody 5·14 mg. (5·7 mg. per ml. of S8).

Main experiment: 5 ml. of S8, 5 ml. of 0·9 per cent NaCl and 1 ml. of 0·395 per cent albumin (3·95 mg.). Albumin added to the washed precipitate 1·8 ml. of 7·90 per cent solution (142 mg.). Total albumin 145·95 (37 times the equivalent amount).

The mixture was left overnight at room temperature. The precipitate dissolved completely. The volume was made up to 8 ml. with phosphate buffer, pH 8·0, $\mu = 0{\cdot}1$; protein concentration 2·20 per cent.

Protein in precipitate, from pilot experiment . .	= 32·5 mg.
Albumin added	=142 „
Total albumin	=146 „
Total protein	=174·5 „
Protein found (8 × 22·0) mg.	=172·0 „

Dialysed against phosphate buffer pH 8·0 for 2 days. Composition by electrophoresis on 3 occasions; the concentrations of the solutions inevitably differed on these 3 occasions:

	Ascending.	Descending.
6.i.50 (2 days after re-solution, Fig. 10):		
Free albumin	78·6 per cent .	83·2 per cent.
Complex and fast component of S8 .	22·4 „ .	16·8 „
11.i.50 (7 days after re-solution, Fig. 10A):		
Free albumin	75·0 per cent .	82·6 per cent.
Complex and fast component of S8 .	25·0 „ .	17·4 „
17.i.50 (13 days after re-solution):		
Free albumin	74·5 per cent .	79·3 per cent.
Complex and fast component of S8 .	25·5 „ .	20·7 „

As the proportions of the fractions did not change appreciably between the 7th and 13th day after re-solution, we assume that final equilibrium had been reached by the 7th day.

In this case it is more satisfactory to calculate the combined albumin from the difference between the complex formed and the precipitable antibody found in the pilot experiment, rather than from the difference between the albumin added and free albumin found, which is a small difference between two large quantities.

6.i.50	Total protein	=174·5 mg.
	Complex (174·5 × 0·224 mg.)	= 39·1 „
	Antibody from pilot experiment	= 28·5 „
	Combined albumin, by difference	= 10·6 „

Ratio of antigen to antibody in complex, $\frac{10{\cdot}6}{28{\cdot}5} = 0{\cdot}37$.

Molecular ratio, 0·85.

11.i.50	Complex (174·5 × 0·25 mg.)	= 43·6 mg.
	Antibody	= 28·5 „
	Combined albumin, by difference	= 15·1 „

Ratio of antigen to antibody in complex, $\frac{15{\cdot}1}{28{\cdot}5} = 0{\cdot}53$.

Molecular ratio, 1·2.

(B) *Addition of antibody solution directly to excess of antigen.*

7 ml. of a more dilute solution of S8, containing 60·9 mg. of globulin, 18·2 mg. of precipitable antibody and 3·5 mg. of inert fast component were added slowly, with thorough mixing, to 0·8 ml. of 7·95 per cent solution of ox serum albumin (63·6 mg.). The solution S8 contained 39·2 mg. of γ-globulin that was not precipitable by antigen.

The mixture was dialysed against phosphate buffer pH 8·0. A precipitate, 2 mg., formed; this differed from the usual antigen-antibody precipitate, as it was not readily soluble in dilute acid.

Composition by electrophoresis (Fig. 13):

	Ascending.	Descending.
Albumin	42·3 per cent	47·8 to 50·4 per cent.
Complex	34·2 „	30·8 to 28·2 „
γ-globulin	23·5 „	21·4 per cent.

Total protein (60·9 × 63·6) mg.	124·5 mg.
Total albumin	63·6 „
Free albumin (124·5 × 0·423 mg.)	52·8 „
Combined albumin, by difference	10·8 „
Complex plus fast component of S8 (124·5 × 0·342 mg.)	42·5 „
Complex (42·5–3·5 mg.)	39·0 „
Globulin in complex	28·2 „

Ratio of antigen to *precipitable antibody* in complex $\frac{10{\cdot}8}{18{\cdot}2} = 0{\cdot}595$.

Molecular ratio, 1·4.

Ratio of antigen to total γ-globulin in complex, $\frac{10{\cdot}8}{28{\cdot}2} = 0{\cdot}38$.

Molecular ratio, 0·87.

(C) *Addition of antigen to supernatant after precipitable antibody had been removed.*

7 ml. of diluted supernatant from Experiment 8A containing 0·42 per cent of globulin (of which 8·5 per cent was fast inert component, Fig. 6A) were added slowly with thorough mixing to 0·8 ml. of 7·95 per cent ox albumin solution (63·5 mg.). The mixture was dialysed against phosphate buffer, pH 8·0.

Composition by electrophoresis (Fig. 16):

	Ascending.	Descending.
Albumin	66·9 per cent	71·4 per cent.
Intermediate component	9·3 „	5·8 „
γ-globulin	23·8 „	22·8 „

Total protein	93·0 mg.
Intermediate component (93 × 0·093 mg.)	8·6 „
Inert fast component of S8 (29·4 × 0·085 mg.)	2·5 „
Complex, by difference	6·1 „
Total albumin	63·6 „
Free albumin (93 × 0·669 mg.)	62·0 „
Bound albumin	1·6 „
γ-globulin in complex	4·5 „

In Fig. 16 there is evidence of a small but definite peak between the peaks corresponding to albumin and γ-globulin.

Experiment 10.

Antigen: Ox serum albumin (Armour); no evidence of α_1-globulin or slower component (Fig. 3).

Antibody: Solution of γ-globulin S9 + 10, which contained no detectable faster component (Fig. 7 and 7A); total protein 10·7 mg. per ml.; precipitable antibody 3·24 mg. per ml.

(A) *Addition of antibody directly to excess of antigen.*

7 ml. of the solution S9 + 10 containing 22·7 mg. of precipitable antibody were added slowly, with thorough mixing, to 0·7 ml. of 8·25 per cent solution of ox serum albumin (17 times the equivalent amount); the mixture was slightly opalescent. On dialysis against phosphate buffer pH 8·0, 0·4 mg. of precipitate formed. This, as in Experiment 9B, was partly insoluble in dilute acid.

Composition by electrophoresis (Fig. 14):

	Ascending.	Descending.
Albumin	32·8 per cent	33·0 per cent.
Complex	35·3 „	36·1 „
γ-globulin	31·9 „	30·9 „

Total protein	136·65 mg.
Total albumin	57·75 „
Free albumin (132·25 × 0·328 mg.)	43·5 „
Combined albumin, by difference	14·25 „
Complex (132·25 × 0·353 mg.)	46·8 „
Globulin in complex, by difference	32·65 „

Ratio of antigen to *precipitable antibody* in complex, $\frac{14 \cdot 2}{22 \cdot 7} = 0 \cdot 63$.

Molecular ratio, 1·45.

Ratio of antigen to total globulin in complex, $\frac{14 \cdot 25}{32 \cdot 45} = 0 \cdot 44$.

Molecular ratio, 1·0.

(B) *Addition of excess antigen to the supernatant after removal of precipitable antibody by precipitation with an equivalent amount of antigen.*

Albumin solution, 0·6 ml., containing 3·3 mg. of albumin were added with stirring to 7 ml. of S9 + 10. After floccules formed the mixture was put in a refrigerator, mixed by inversion on the following day, centrifuged on the 3rd day, and the precipitate was washed 3 times with cold saline. It contained 26·0 mg. of protein (22·7 mg. of antibody).

The supernatant fluid was dialysed against phosphate buffer pH 8. No component faster than γ-globulin was detected by electrophoresis (Fig. 17); all the albumin was precipitated and no soluble complex formed.

Ox serum albumin, 0·50 ml. of 8·25 per cent solution (41·25 mg. of albumin) as added to 7 ml. of the dialysed supernatant, which contained 35 mg. of γ-globulin. The mixture was dialysed against phosphate buffer pH 8. A small precipitate (about 0·05 mg.) settled out.

Composition by electrophoresis (Fig. 17A):

	Ascending.	Descending.
Albumin	52·5	54·5
Complex	5·1	4·4
γ-globulin	42·4	41·7

There was a definite peak between those corresponding to albumin and γ-globulin as in Fig. 16.

There was also a shoulder on the γ-globulin peak as in Experiment 8A (Fig. 15). There is direct evidence of the formation of a small amount of complex. The amount is of the same order as the amount in Experiment 9C.

Experiment 11.

There is evidence in the preceding experiments that ox and horse serum albumin form a complex with the γ-globulin of a homologous antiserum from which all the precipitable antibody has been removed. An experiment was therefore made in which ox serum albumin was mixed with γ-globulin (S11) prepared from the sera of 2 rabbits which had not been immunized against ox serum albumin. One of these had received injections of Bence-Jones protein, but had formed no antibody detectable by this antigen; the other had been immunized with crystalline urease and had formed a small amount of precipitable antibody. This γ-globulin (Fig. 19) contained a small amount of a faster component. Of the solution S11 3 ml. (81 mg.) were added, with stirring, to 0·8 ml. of a 8·0 per cent solution of ox serum albumin (64 mg.). The mixture was dialysed against phosphate buffer pH 8·0. The composition by electrophoresis (Fig. 20) was:

	Ascending side.
Albumin	44 per cent.
Globulin	56 ,,

A hump on the slow side of the albumin peak suggests the formation of a small amount of a complex, with a mobility slightly less than that of albumin and therefore containing a high proportion of albumin to globulin. This complex would amount to about 5 per cent of the total protein present. The small fast component of the S11 appeared unchanged; there is no evidence of an intermediate peak comparable to those in Fig. 16A and 18.

DISCUSSION.

In these experiments two matters not directly related to the valency of antibodies may be noted. In the first place, in all the attempts at re-solution, except that of Experiment 9A, the precipitates dissolved much more slowly, and much more antigen was needed to effect solution than in the experiment of Heidelberger and Pedersen (1937). In other experiments which we have not reported we were not able to re-dissolve a considerable proportion of the original precipitate. This difficulty of re-solution was not a characteristic of the antigen concerned, as we had the same difficulty in experiments in which the antigens were human serum albumin and crystalline egg albumin, the antigen used in the experiments of Heidelberger and Pedersen (1937).

In the second place, the antigen-antibody complex persisted in the upper part of the descending limb of the electrophoresis apparatus, although it was left behind by the antigen, which was the faster component; that is, the complex remained, although it was not in presence of excess antigen. In all cases the

amount of complex (calculated from the electrophoresis pattern) was slightly less on the descending side than on the ascending side. It appears that the dissociation of the complex on removal of the antigen was slow. Samples of the complex, with no free antigen, were removed from the descending limb and kept frozen for several weeks; no precipitate formed while the solutions were frozen, but formed in the course of a few days when the solutions were thawed and kept at 4° C. One such sample examined in the ultracentrifuge shortly after thawing by Dr. Kekwick of the Lister Institute was found to be polydisperse; the excess antigen was probably dissociating from the complex and precipitable complexes were being formed.

Although Heidelberger and Pedersen (1937) found that their precipitates dissolved much more readily than ours did, they found some evidence of a progressive change after solution. This can be compared with the change found in our Experiment 9A, in which the amount of antigen in the complex increased between the 2nd and 7th day after re-solution. These slow changes are comparable to the Danysz effect, and contrast with the rapid reversibility, in the region of antibody excess, found by Marrack (1938).

According to the lattice theory the solution of the antigen-antibody precipitate in excess of antigen involves the breaking of large complexes A_mG_n, through intermediate stages, to the final compound AG_2 with possibly some larger compound such as A_2G_3. If, on the other hand, antibodies are univalent, the process of solution only involves the dispersal of the precipitate and the change:

$$A_nG + (n - 1)\,G = nAG, \text{ or } A_nG + (2n - 1)\,G = nAG_2.$$

In either case no process of dispersal or break-up is involved when antibody solution is added to excess of antigen.

In the experiments of Heidelberger and Pedersen (1937) final equilibrium was not reached in 3 days, although the precipitate dissolved readily in excess of antigen; in our Experiment 9A in which the precipitate dissolved overnight there was some change between the 2nd and 7th day after solution, though none between the 7th and 13th days. It might be argued that in Experiments 3 and 6 the final stage of breakdown of large complexes with formation of small compounds containing a higher ratio of antigen to antibody had not been reached. If this were so, there should be evidence of a series of compounds of intermediate composition, and with mobilities intermediate between those of the main complex and of γ-globulin; the complex peak should slope off gradually on the slow side. Actually the slope of the complex peak (Experiment 3 and 6, Fig. 8 and 9) was steep on the slow side, and if anything it was less steep in Experiment 9A (Fig. 10A), in which solution was rapid and no change appeared between the 5th and 11th days after re-solution. Also, when antibody solution was added directly to excess of antigen the break-up to large complexes would not be involved. But the peaks of the soluble complex found in the direct Experiments 7A and 9B (Fig. 11, 13) were very similar to those found in the re-solution experiments; in both there were two peaks, and the demarcation on the side of slower mobility was sharp. In Experiments 3 and 6 (Fig. 8 and 9) there was evidence of a small amount of material of mobility between that of the soluble complex and that of γ-globulin. We suggested in discussion of Experiment 6 that this had the composition AG. It may have consisted of compounds of intermediate composition which would have disappeared if final equilibrium had been reached.

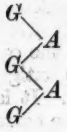
In the Experiments 7, 8, 9B, 10, in which antibody solution was added directly to excess of antigen, the amount of globulin found in the soluble complexes was considerably more than the precipitable antibody found in the antibody solutions. This complicates the calculation of the ratio of antigen to antibody in the complexes and these experiments will be discussed later. In Experiment 3, 6 and 9A in which the precipitate was re-dissolved the molecular ratio of antigen to antibody in the complex was more than 1 and less than 2. The ratios in Experiment 2, 4, 5 and 8, as far as it was possible to calculate by making allowance for the undissolved precipitate, were also between 1 and 2.

A remarkable feature of all the electrophoretic patterns—except Fig. 14 (Experiment 10A)—was the presence of two peaks corresponding to the antigen-antibody complex. In Fig. 8, 9 and 11 these peaks were sharply demarcated. The presence of these 2 distinct peaks suggests that 2 distinct compounds were formed. The most obvious explanation is that the two components were AG_2, formed by bivalent antibody, and AG, formed by univalent antibody. In Experiment 3 the molecular ratio in the whole complex was 1·7 or 1·8; to fit this ratio the ratio of molecules of AG_2 to molecules of AG would be 70 to 30 and the ratio of the corresponding areas of fast and slow components would be 210 to 69; the ratio of the corresponding areas (Fig. 8) is 19 to 34. In Experiment 6, again, the molecular ratio in the whole complex was 1·6; the corresponding ratio of molecules of A_2G to molecules of AG would be 60 to 40 and the ratio of the corresponding areas would be 180 to 92; the ratio calculated from Fig. 9 is 14·6 to 15·5. The differences in these two instances, in which the two peaks were clearly separated, is greater than the expected error of the estimates. The slower component of the complex must therefore have a composition A_mG_n, intermediate between AG_2 and AG.

In Experiment 6 a mixture of 10 molecules of AG_2, 6 of A_2G_3 and 1 of AG would fit the ratio of fast and slow components and the ratio of antigen to antibody found in the total complex; but this is not the case in Experiment 3 (Fig. 8), in which the area of the peak corresponding to the slower component of the complex is considerably larger than that corresponding to the faster component. Two peaks were found in Experiment 9A (Fig. 10A) in which final equilibrium was reached, and in Experiment 7A (Fig. 11) in which the breakdown of the large complex was not involved. So the presence of the slower peak with the composition A_mG_n cannot be ascribed to incomplete breakdown of larger molecules.

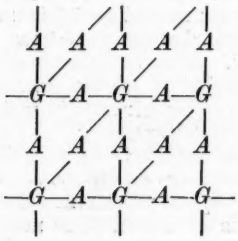
In Experiment 6 the molecular concentration of the free antigen is about 8 times the molecular concentration of the antibody. If no other factor besides the specific valencies of antibody and antigen were involved it would be expected, on grounds of probability, that most of the molecules of antigen would be combined with one molecule of antibody only; and that if antibody molecules have two valencies the bulk of the compounds formed would be AG_2 or AG, the ratio of the two concentrations, depending on the dissociation constant; there is, however, evidence of molecules of the composition A_mG_n. Another factor involved may be a non-specific attraction between antibody molecules when these are brought into close apposition by specific combination with antigen, as suggested by Boyd (1947). It is not necessary to regard compounds such as A_2G_3 as a chain. It is not improbable that antibody molecules may have two valency groups placed asymmetrically, as they seem to be on the molecules of diphtheria

antitoxin (Pappenheimer *et al.*, 1940). The compound A_2G_3 may be more appropriately represented by—

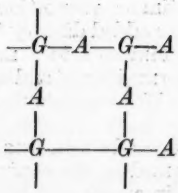


When the lattice theory was first suggested (Marrack, 1934) it was pointed out that, when antibody is in great excess, all the valencies of each antibody molecule are satisfied without any antibody molecules combining with two antigen molecules; and that, consequently, large complexes cannot be built up by specific valencies. It was then suggested that "when antibody molecules are attached to the antigen, the polar groups, on which the solubility of the antibody globulin normally depends, are brought into close apposition with each other and attract each other instead of water molecules."

We may therefore suggest that the second valency of antibody molecules is weak and is reinforced when antibody is in excess, and to a lesser degree, when antigen is in excess, by this non-specific attraction. In the neighbourhood of the equivalence zone the force holding a pair of molecules of antigen to one molecule of antibody is also reinforced by the attractions between other molecules of antigen and antibody. The stability of the structures depends on the fact that antigens are multivalent, so that structures such as—



are possible. Structures such as—



would be relatively unstable. Hence the formation of a precipitate is inhibited when antigen is in excess. The stability of the complex depends on the formation of a framework with several ties. The term "lattice" may be unsuitable, as the word has other connotations, and might be replaced by "framework." But "alternation" is unsuitable, as it suggests a linear structure.

The antigens used in these experiments were not homogeneous ; even the ox albumin may have contained some 2 per cent of a component which had a sedimentation constant above that of albumin. In all cases the antibody solutions were tested by the optimum proportions method of Dean and Webb (1923), with antigen solutions in amounts up to 5 times and down to one-third of the optimum. No zone was found other than that due to the major constitunet of the antigen ; this zone corresponded to the equivalence zone of the precipitation curve. It is possible that the reaction between a minor antigen and a small amount of the corresponding antibody might be hidden in the major zone. No precipitate was formed when the supernatant fluids at the equivalent points were tested with further antigen.

Solution S6 (S4 and S6 were prepared from the serum of the same rabbit, bled at different times) was tested by Oudin's (1947) method. When a solution of horse serum albumin (Fig. 2) was put on the agar column, a faint zone appeared above the main zone. When horse serum was superimposed a faint zone appeared below the main zone. It appears, therefore, that solution S6 contained a small amount of antibody to an antigen of which there was much less in the albumin solution than in the whole serum. In Experiment 7A, 7B, 8, 9B and 10A, in which antibody solutions were added directly to excess of antigen, the amount of globulin found in the complexes was 37 to 50 per cent more than the precipitable antibody. The precipitable antibody in these experiments was estimated by adding the equivalent amount of antigen to the antibody solution in one lot ; it included any incomplete antibody, such as that demonstrated by Heidelberger and Kendall (1935), which would be carried down with the precipitate formed by the complete antibody present. A minor constituent (G') of the antigen may have contributed towards this excess. The contribution cannot, however, account for the excess found. In Experiment 9B, 63·6 mg. of antigen (ox serum) albumin was used ; at 2 per cent this would contain about 1·3 mg. of G'. If *all* this was combined with antibody to form a soluble compound (which is improbable) and the composition of this compound was AG_2, this would amount to 3·9 mg. ; whereas the excess of globulin found in the complex was 10 mg. Also, if the excess of globulin found in the complexes in these experiments were included in soluble compounds of G' and anti-G', these complexes should also be found in experiments in which excess of antigen was added to supernatants after the precipitable antibody had been removed. No such complex was found in Experiment 8A, but definite peaks were found in Experiment 9C and 10B. In neither case were these peaks large enough to account for the excess found in 9A and 10A, although the accuracy of the measurements involved may not be enough for a conclusion to be based on this difference. The main reason for not attributing the excess of globulin in the complexes in these experiments to the presence of antibody to minor constituents of the antigens is that if a sufficient amount of such antibody had been present it should have been detected in the supernatant fluids, formed at the equivalence point, and by the optimum proportions method. In the experiments, other than 7A, in which antibody was added directly to excess of antigen, some precipitate, ranging from 0·4 mg. in Experiment 4 to 4 mg. in Experiment 8, was formed. These precipitates may have been formed by G' and anti-G', though the relative insolubility of these precipitates in dilute HCl suggests that they were not genuine antigen-antibody precipitates.

It seems, therefore, that the antibody solutions contained globulin that was not carried down in the precipitate formed at the equivalence point, but would combine with antigen when this was added in excess. It is quite possible that the amount of incomplete antibody that can be precipitated with the complete antibody is limited and the amount in an antibody solution may exceed the amount that can be carried, down in this way.

The estimates suggest that this excess globulin, that combined with antigen when whole antibody was added to excess of antigen in Experiments 9B and 10A, was more than the amount of globulin that combined when the supernatant fluids were added to

excess antigen. If so, some of this incomplete antibody was incorporated in complexes formed by precipitable antibody with excess of antigen, but did not combine with antigen when precipitable antibody was not present. This incorporation may have depended on the non-specific attractions between antibody molecules, as well as on a weak specific affinity for antigen. When the amount of antigen was less all the combining sites of antigen could be occupied by precipitable antibody, and this antibody of weak affinity would be excluded.

The excess globulin found in the soluble complexes in the experiments in which antibody solutions were added to excess antigen complicates the calculation of the ratio of antigen to antibody in these complexes. In Experiment 7A the molecular ratio of antigen to precipitable antibody was 2·0, but the ratio of antigen to total globulin was 1·34. If the excess globulin was incomplete antibody, with which some of the antigen was combined, it should be taken into account and conclusions on the valency of the antibody should be based on the lower ratio.

SUMMARY.

(1) The composition of the compounds formed by antibodies with excess of the homologous antigens has been studied by electrophoresis.

(2) Evidence has been found of the formation of two compounds of different composition.

(3) Antibodies appear to be bivalent.

(4) It is suggested that the formation of large complexes by antigens and antibodies depends on the formation of a framework in which a pair of antigen molecules are held together by several links; also that non-specific attractions between antibody molecules may increase the stability and insolubility of the complexes.

(5) Evidence has been found of the presence in solutions of γ-globulin, that contain antibodies, of some globulin that can be incorporated in soluble antigen-antibody complexes, but is not carried down with precipitable antibody in the equivalence zone.

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FACTORS INFLUENCING THE DEVELOPMENT OF POTENTIAL IMMUNITY AND THE CHARACTER OF THE SECONDARY RESPONSE.

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IN 1931 Glenny stated: "Antigens suitable for human use have not yet been produced of sufficient potency to immunize successfully by means of a single injection. The power of an antigen therefore to induce high immunity in a guinea-pig after a single dose is not necessarily an index of its value for human immunization, but is of use in comparing the relative strengths of different batches of the same preparation." After a lapse of twenty years these observations still appear to hold good.

The method used for testing a batch of antigen should depend on the purpose for which the material is intended. Antigens may be used (1) for the hyper-immunization of horses in the production of therapeutic sera, and (2) for the prophylactic immunization of animals and man. Group 2 may be further subdivided into prophylactics intended to establish basal immunity, and those required to boost antitoxic levels that have fallen with the passage of time. It is possible (Barr and Glenny, 1951) that the ideal course of human immunization should include the use of more than one type of prophylactic.

Carlinfanti (1950) stated that "the two-dose method is totally inadequate to reveal quantitatively the difference between a good and a poor prophylactic." In his work he was comparing different types of diphtheria prophylactic, to which the primary responses could not be expected to be comparable. In contrast to this, Barr and Glenny (1949), in work restricted to the comparison of crude diphtheria formol toxoids, failed to find a satisfactory single dose that would detect differences so well as the two-injection method.

Most workers interested in the prophylactic immunization of man against diphtheria and tetanus would agree with the aims of active immunization suggested by Barr and Glenny (1951). These include the establishment of sound potential immunity, the production of good antitoxic titres, and the maintenance of some antitoxin in the circulation for many years after the course of prophylactic injections. We are of the opinion that at the present time this state of affairs cannot be brought about in man by a single injection of any type of prophylactic. It therefore appears to us that, unless strong evidence to the contrary is forthcoming, Carlinfanti's one-injection method should not be used as a routine test for preparations intended for human immunization. In our experience a single-dose method may be of great value when used for the purpose suggested by Glenny (1931), but it does not measure the degree of potential

immunity established. We have found (unpublished) that a very small dose (0·05 Lf dose) of an adsorbed diphtheria prophylactic may confer excellent potential immunity in guinea-pigs that at no time showed detectable circulating antitoxin after the injection. This potential immunity was demonstrated by the appearance of 1 unit or more of antitoxin per ml. in the circulation of most of the animals 10 days after a second injection given as long as seven months after the first. Individual results were very variable, however, and some animals gave a poor response. This result, together with others we are recording in this paper, suggests that potential immunity had declined in the poor responders during the long interval between the two injections.

While a temporary advantage is gained in prophylactic immunization by antitoxin-production after a single injection of prophylactic, it appears to us that so long as additional injections are needed, the establishment of basal immunity should be regarded as the most important function of the primary stimulus. A good secondary response should follow the second injection, and in order to ensure this the first dose should be relatively large.

Experience in hyperimmunization (Barr and Glenny, 1945) has shown that potential immunity increases long after antitoxin-production has declined following prophylactic injections. This conclusion was drawn from the fact that in the production of tetanus antitoxin, horses given preliminary injections many months before their hyperimmunization course produced antitoxin of better quality and higher value than those given a shorter resting period. (The term " quality " refers to the degree of avidity as measured by the serum ratio *in vivo* value/*in vitro* value : a low value for this ratio indicates loose combination of the antitoxin with toxin, so that dissociation occurs *in vivo*).

The experiments recorded here show that the amount of antitoxin produced from primary stimulation may bear little relation to the degree of potential immunity established by different prophylactics, and that if too long a delay is left between the first and second injections, potential immunity, judged by values 10 days after a second injection, may decrease. This suggests that the spacing of injections and of boosting doses is of great importance in human immunization, and it appears reasonable to suppose that there exists an optimal interval, most suitable for the majority of persons, which would be dependent on the nature of the prophylactic and the dose used in relation to body-weight.

Antitoxin-production in guinea-pigs after primary stimulation with diphtheria prophylactic.

Two groups, each of eighty guinea-pigs, were injected subcutaneously, the one with 1 Lf dose of P.T.A.P., and the other with 1 Lf dose of A.P.T. The P.T.A.P. was a routine matured batch containing 60 Lf doses and 10 mg. $AlPO_4$ per ml. All injections were made in a dose of 1 ml., the material having been suitably diluted in normal saline. The animals were bled at monthly intervals after injection, until they were due for re-injection in the second part of the experiment. All blood samples in this work were titrated individually for antitoxic content at approximately 2-fold differences : the guinea-pig intracutaneous method was used (Glenny and Llewellyn-Jones, 1931), and at least two tests giving the same end-point were made on each sample.

In this particular experiment individual guinea-pigs were not distinguishable. This fact limits the conclusions that we were able to draw, because although the

scatter of values was determined, it is not known whether all guinea-pigs reached the height of their response at the same time. The differences between the values of the P.T.A.P. group and the A.P.T. group were sufficiently great to justify the

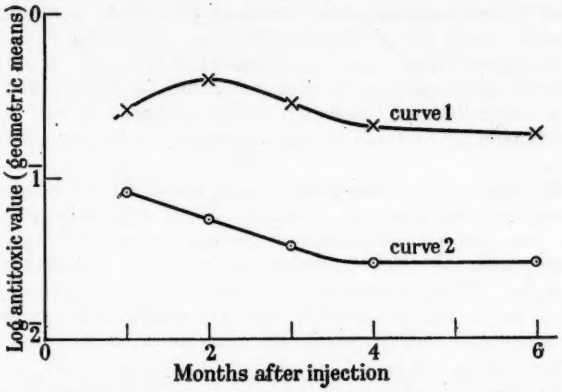

FIG. 1.—The response of guinea-pigs to a single injection of diphtheria prophylactic (geometric means).
Curve 1 : 1 Lf dose of P.T.A.P.
„ 2 : 1 „ „ A.P.T.

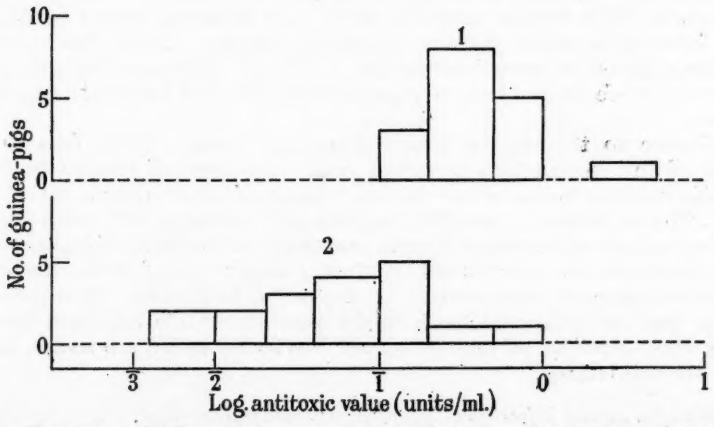

FIG. 2.—The scatter of values of guinea-pig sera 2 months after a single injection of diphtheria prophylactic.
(1) 1 Lf dose of P.T.A.P.
(2) 1 „ „ A.P.T.

comparison of response curves for the two groups. These are shown in Fig. 1, where the logarithms of the geometric means are plotted against time after injection. The peak was reached with P.T.A.P. at two months and that for A.P.T. at one month or earlier, though we are unable to say that all animals reached their peak at these respective times. The values of guinea-pigs injected

with P.T.A.P. were very appreciably higher than those of animals injected with A.P.T., and the scatter of values at two months is shown for both groups in Fig. 2. At this stage the geometric mean for the P.T.A.P. group was at the peak, and that for the A.P.T. group had fallen slightly. It will be seen that the values of the former showed a small scatter, and those of the latter a much larger scatter with a tail below the peak of the distribution. A similar type of scatter was observed at all times when blood samples were titrated. The evidence from Fig. 1 and 2 is in agreement with that presented by Holt (1950*a*) for primary responses to a larger dose of these two prophylactics. It will also be noted in Fig. 1 that a flattening of both curves appeared to be occurring four months after injection.

Holt (1949), apparently using pooled sera, obtained a " baseline " or steady level three months after a single injection of P.T.A.P. in guinea-pigs. In our experience in the tracing of primary response curves in individual but not distinguishable guinea-pigs, falls in value may continue to occur in some animals after four months. This conclusion was drawn from the values obtained from groups in which no deaths occurred in the later stages of the experiment : most of such groups are observed by us for six months after injection. Holt (1949) quotes the observations of Glenny and Sudmersen (1921) in an experiment in which a single dose of toxin-antitoxin mixture was injected subcutaneously into guinea-pigs, the antitoxic titres of which were followed for a period of two years and " for the greater part of the latter period of observation the animals showed a constant titre of circulating antitoxin—the final baseline of immunity." He continues : " Glenny and Barr (1947) have reported a similar phenomenon in horses in which tetanus antitoxin titres were observed over a period of five years following a single injection of tetanus toxoid." Since this observation concerns a matter of some fundamental importance in connection with primary stimulation, we feel it necessary to point out that this work has been misinterpreted by Holt.

Reference to the original paper (Barr and Glenny, 1947) fails to reveal records of any experiments involving single injections of tetanus toxoid, nor were observations made on the antitoxic titres of horses over a period of five years. The experiments recorded by Barr and Glenny (1947) were concerned with the amounts of tetanus antitoxin remaining in the blood of *hyperimmunized* horses about *one year after the last injection of toxin :* one of the horses had been hyperimmunized with tetanus toxin for five and a half years. It is presumably possible that an occasional horse might have detectable antitoxin five years after a single injection of tetanus toxoid, but so far as we are aware, evidence to this effect is lacking.

The effect of a second injection of prophylactic into guinea-pigs.

The groups of guinea-pigs used in the first part of the experiment were each subdivided into four distinguishable groups and re-injected with the same dose of the same prophylactic, but at different times after the first injection. These intervals were 1, 2, 3 and 6 months. The animals were bled 10 days and 1, 2 and 3 months after re-injection and the individual sera titrated for antitoxic content.

The guinea-pigs were not individually distinguishable, but the scatter of values within the groups was small, and response curves have been plotted using the geometric means ; these are shown in Fig. 3.

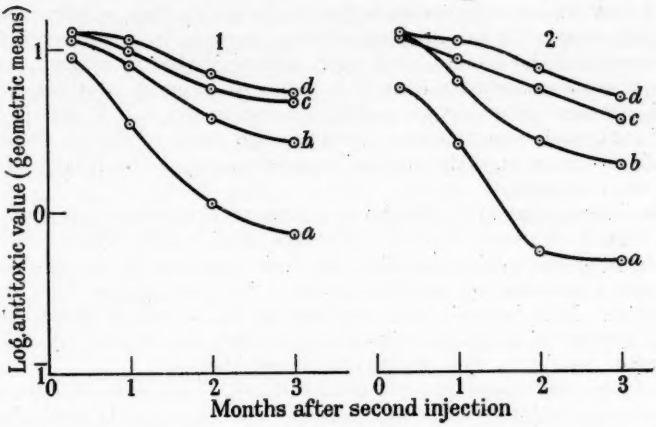

FIG. 3.—The response of guinea-pigs to a second injection of diphtheria prophylactic (geometric means).

(1) Both injections 1 Lf dose of P.T.A.P.
(2) „ „ 1 „ „ A.P.T.

(*a*) 2nd injection 1 month after 1st.
(*b*) „ „ 2 months „
(*c*) „ „ 3 „ „
(*d*) „ „ 6 „ „

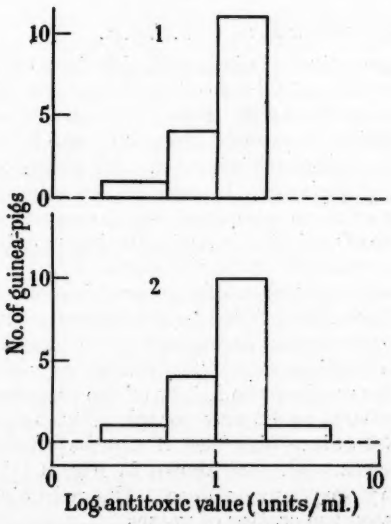

FIG. 4.—The scatter of values of guinea-pig sera 10 days after the second injection of prophylactic given 6 months after the first.

(1) Both injections 1 Lf dose of P.T.A.P.
(2) „ „ 1 „ „ A.P.T.

A remarkable similarity exists between the responses to the second injection of comparable groups injected with the two prophylactics, whatever the interval between injections. It will be noted that an increase in this interval from 1 to 2 months increased the 10-day response: with a further increase in interval this response was not affected within the limits of experimental conditions. The effect of increased interval was slightly greater in the A.P.T. group. A further point of interest is the difference between the rates of fall in value from the height of the response. The rate of loss decreased as the interval between injections was increased.

These observations apply to the responses to the second injection of animals injected with both prophylactics. Whereas considerable differences in response between groups were observed after the first injection, a remarkable similarity was apparent between comparable groups after re-injection with the different preparations. Evidence of this is provided in Fig. 4, which shows the scatter of values in groups of 16 guinea-pigs 10 days after the second injections given 6 months after the first. The results are almost identical.

This experiment shows that potential immunity (judged by the 10-day response to the second injection) may be maintained for a considerable time after the peak of a primary response. It also shows that with a dose of 1 Lf, the potential immunity existing over the period of observation was in general unrelated to the degree of antitoxin-production provoked by the first injection (Fig. 1 and 3). It is probable that potential immunity would eventually decline, but it is not practicable to house and re-inject guinea-pigs over a period of several years. In the next experiment we therefore used P.T.A.P. in smaller doses.

The effect of injection of smaller doses of P.T.A.P.

A group of 100 guinea-pigs, all distinguishable from one another, were injected subcutaneously, each with 0·25 Lf dose of the same batch of P.T.A.P. as that used in the preceding experiment. They were subdivided into five groups according to the interval fixed before the second injection: the intervals chosen were 1, 2, 3, 4 and 6 months after the first injection. All guinea-pigs were bled and the individual sera titrated for antitoxin content immediately before re-injection: three groups had one or more additional blood samples taken. This was done to determine the degree of variation in antitoxic titre and form of primary response curves for different animals treated in the same way.

Fig. 5 shows the antitoxic values of 9 guinea-pigs bled 1, 2 and 3 months after the first injection. One animal failed to produce any detectable antitoxin over the period examined, one reached its highest titre 1 month after injection and the remainder 2 months after injection. The scatter of values at 2 months, when most of the guinea-pigs reached the height of the response, was relatively large, extending from under 0·001 to 0·2 unit per ml. Fig. 6 shows the scatter of antitoxic values among 32 guinea-pigs bled 2 months after the first injection. A comparison of this result with that shown in Fig. 2 (1) clearly illustrates the dependence of primary response on dose. The reduction of the dose to one-quarter greatly increased the scatter of values.

Table I gives the antitoxic values of all guinea-pigs surviving for the duration of the experiment, at the time of receiving the second dose of prophylactic and at fixed chosen times thereafter. The animals are grouped according to the time at

which the second injection was given, and arranged in order according to their antitoxic values 10 days after the second injection.

An excellent degree of uniformity existed among the 10-day values of the guinea-pigs in Group 3. This suggests that there was but little variation in the degree of potential immunity in individuals 3 months after the first injection of 0·25 Lf dose. The scatter of 10-day values was greater in the other groups, and while some of those in Group 4 were higher, others were lower than those of Group 3. This suggests that a further increase in potential immunity occurred in some animals between the third and fourth month after the first injection : in a few there was a decrease. A general decrease appeared to have occurred by the sixth month.

There appears to be a rough correlation between the values produced by individual guinea-pigs after the first and after the second injection. The guinea-pigs in Groups 2, 3 and 4 were all bled two months after the first injection, and a comparison between the values of these samples and those of the same animals 10 days after the second injection is given in Table II. Although the numbers are small, it appears that the majority of animals reaching low values after the first injection did not respond so well to the second injection as did those producing higher values after the first injection.

A striking point emerging from the data in Table I is the variability in the amount of antitoxin lost over the period observed after secondary stimulation. In general the fall in value among good responders, from 10 days to 6 months, decreased as the interval between injections was increased, and was extremely small in some of the animals in Group 5. Owing to the wide limits of our tests rates of loss cannot be calculated, but it is perfectly clear from the figures that many differences in individual rates are well outside the limits of error of the experiment. This is illustrated in Fig. 7, discussed at a later stage : the limits of error in the allocation of values have been shown in plotting these curves.

The loss of antitoxin in the poorer responders cannot be correlated with interval between injections, but appeared to be greater in those animals that had no detectable circulating antitoxin at the time of the second injection. It is of interest to note that the absence of antitoxin at this time does not mean that no antitoxin had ever been detected in these animals after the first injection. All but one had produced antitoxin at an earlier stage. This suggests that the power of continued production is poor in some animals after both the first and the second injection.

Holt (1949) considers that " pure primary response " curves show no peak and that any peaking with time, observed after a single injection, is due to a secondary response effect superimposed on a " pure " primary. He claims to have shown that this peak falls to a base-line value at the same level as that obtained in the " pure " primary response curve. If we accept this hypothesis, then those of our guinea-pigs that had circulating antitoxin 2 months after the first injection and subsequently lost it (4 months after injection) must have produced this antitoxin *entirely* as a result of a secondary response effect. Secondary responses are frequently observed after a second injection of antigen into animals that have produced no detectable antitoxin after the first injection. In this experiment, however, we have obtained what is termed by Holt (1950*b*) a " pure secondary response " following the first injection of a very small single dose of prophylactic into non-immune guinea-pigs.

We agree with Holt's (1949) idea that primary responses are made up of component effects, one of which is similar to the secondary response and is caused by residual antigen. Glenny, Buttle and Stevens (1931) visualized the effect of an injection of antigen into a normal animal as a process of continual stimulation by lessening quantities of antigen, acting on tissues whose power of response is rapidly increasing.

It appears, however, that the terms " pure " primary response and " pure " secondary response are unfortunate. There is no evidence to show that a " pure primary response " as conceived by Holt (1949) is not made up of two factors, one

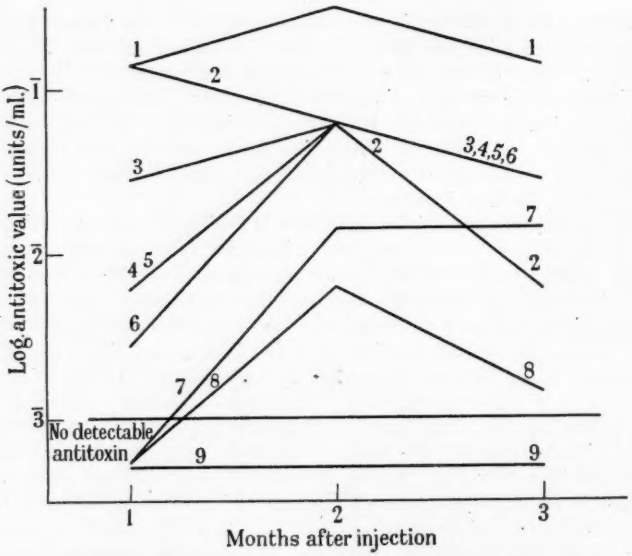

FIG. 5.—The antitoxic values of individual guinea-pigs bled at monthly intervals after a single injection of 0·25 Lf dose of P.T.A.P.

the sensitization of cells or production of potential immunity, and the other the formation of antitoxin. It is departing too far from the essential meaning of the words " primary " and " secondary " to describe the first observable effect after primary stimulation as a " pure secondary response."

The testing of immunity by antitoxin titrations.

In the experiments we have described, individual sera have been tested at approximately 2-fold differences, and confirmatory tests made. Values recorded in Table I, as for example 5 units, are in fact over 5 but under 10 units. In calculating the geometric means of groups and in constructing Fig. 5, the values of individuals were taken as the geometric means of the limits between which they lay ; this means that a value given as 5 units in Table I was taken as $\sqrt{5 \times 10}$ ($= 7{\cdot}07$) in the calculation of geometric means.

The volume available for testing the antitoxic value of serum from a guinea-pig is small, and may not suffice for close and accurate testing : the values allotted are not absolute because such sera are frequently non-avid, (Glenny, Barr, Ross and Stevens, 1932 ; Jerne and Maaløe, 1949). The methods used by us have the advantage, however, of revealing individual variations in value and type of response among the animals of a group.

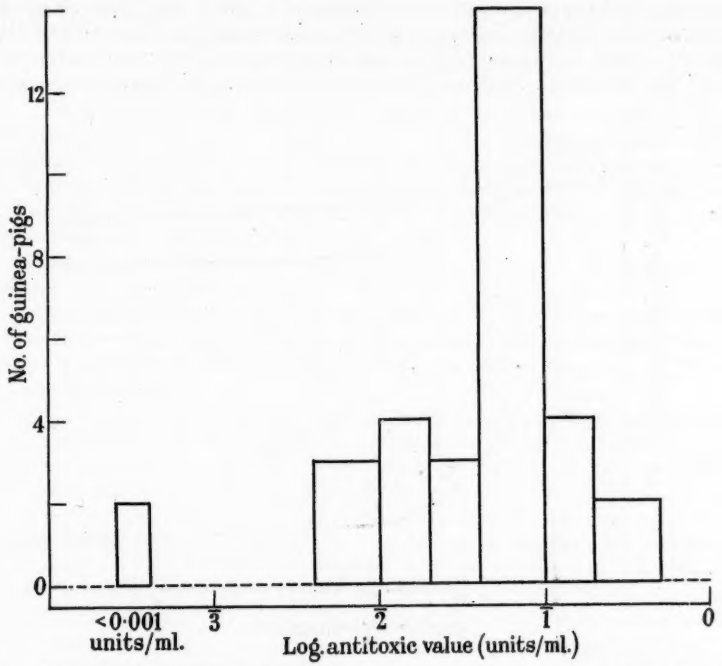

FIG. 6.—The scatter of antitoxic values of sera among 32 guinea-pigs 2 months after a single injection of 0·25 Lf dose of P.T.A.P.

It is unfortunate that conclusions of a fundamental nature have been drawn by some workers on the results of tests on pooled samples. The pooling of samples provides sufficient material for close accurate tests to be made, but individual variation is quite unknown, and essential information is thereby lost ; as a result unjustifiable conclusions may be drawn.

Faragó and Pusztai (1949), using pooled sera from rabbits injected with a combined diphtheria-tetanus-pertussis prophylactic, claimed to have obtained a two-stage secondary response to the tetanus constituent ; the antitoxic value did not rise in the usual uninterrupted manner to the peak of the response. Glenny, in his Sir Almroth Wright Lecture (May, 1950), pointed out that such a two-stage secondary response could apparently be obtained by plotting the arithmetic means of values of the sera of horses that did not reach the peak of the response

at the same time after injection. While it is not denied that a two-stage response might conceivably occur, no such phenomenon has been observed by us in individual animals, and we suggest that the unexplained result of Faragó and Pusztai may have been obtained fortuitously, from the testing of pooled sera from animals that responded in a dissimilar manner.

While the values obtained for pooled sera may represent the average for a group, it is also possible that they do not represent the value of any single member of the group, and one abnormally high value may raise the mean so excessively as to convey an entirely false impression of the whole. More especially is it dangerous to work out quantitative data from response curves constructed from values of pooled serum bled on different occasions. If the animals concerned

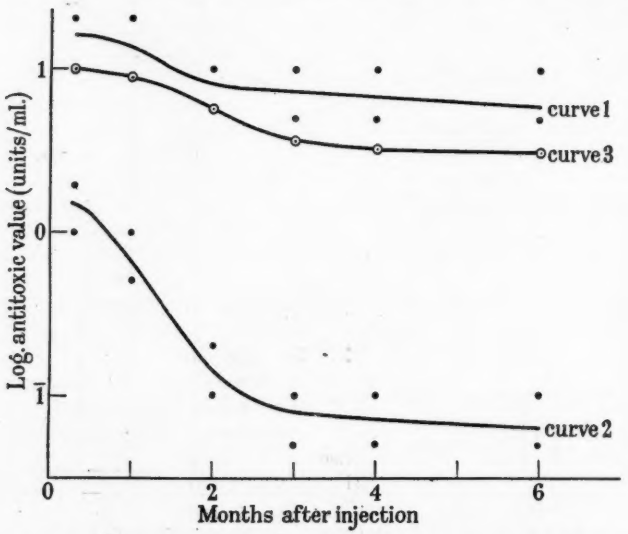

FIG. 7.—Secondary response loss curves taken from Group 5 in Table I.
Curves 1 and 2 responses of individual guinea-pigs.
Curve 3 values of the arithmetic mean for the whole group.
Vertical dots give the limits within which individual observations lie.

reached the peak of their response at different times, and the subsequent rate of loss varied in the group, a curve might be constructed which was truly representative of none of them. Thus a curve constructed from the arithmetic means of a number of individual curves, all showing sharp rises and falls, might show a gradual rise to a peak maintained for a considerable time if the individuals reached their peaks at different times. The information lost in pooling sera in the tracing of response curves includes (1) the scatter of values and (2) the configuration of individual curves: both these are of the utmost importance. Owing to the relatively sharp rise and fall of many secondary response curves, a knowledge of the second factor is usually more important in the tracing of such curves. The chief variable in primary responses is frequently the scatter of values, but the effect of both factors can be seen for primary responses in Fig. 5. Only when

individual variation is known can the limitations of conclusions to be drawn be appreciated.

Fig. 7 shows the fall from the secondary peaks of the first and last guinea-pigs in Group 5, Table I; the figures in Table I show that the first 3 guinea-pigs in this group had the same individual values at the various times of sampling, and that the titres of the last 2 fell in a comparable manner. In constructing curves 1 and 2 in Fig. 7, points were plotted for the limiting levels in the antitoxin titrations (i.e., guinea-pig 1, value recorded as 10 units, points plotted were log 10 and log 20) and curves drawn between the appropriate points. Curve 3 was constructed by plotting the logs of the arithmetic means for the whole group. In calculating arithmetic means, values of over 10 and under 20 were taken as 15 units. This curve would have been obtained if pools consisting of equal volumes of individual sera had been titrated. In the absence of a knowledge of the individual values, this curve might have led us to conclude that a very high degree of immunity existed in all the animals of this group. The mean value 6 months after the second injection was 3·22, and the magnitude of this figure might be taken as indication of a small scatter.

Holt (1950*a*) used pooled sera extensively in some of his experiments. This fact, together with the absence of any mention of procedure, or scatter of values, and the actual figures (e.g., 1·0, 1·5) recorded in tables, renders it practically certain that pooled sera were titrated in at least some of the experiments described in his other publications (1949, 1950*b*); in a few tables the values given are headed "average values."

Holt (1950*b*) considers that the secondary response phenomenon of Glenny and Sudmersen (1921) is the resultant of three separate components, (a) the initial serum titre, (b) the secondary response effect or "leap," and (c) the contribution of the secondary stimulus towards the new base-line level of immunity. In this quotation we have corrected Holt's misleading expression "second" and reverted to the original "secondary" response, because the use of the word "second" seems to imply the response to a second injection. As originally stated by Glenny and Sudmersen (1921), an intermediate response is obtained on injecting an animal that is only partially immune, and could thus follow a second injection of antigen into an animal that had received an extremely small first injection. The term "secondary response" has no bearing on the number of injections given, but is used to describe the *type* of response given after the stimulation of animals in which basal immunity is well established. It would thus be shown after the tenth or fiftieth injection of prophylactic.

Most workers will agree with Holt's general conception of the secondary response, because its magnitude is measured in terms of increased titre (although some workers unfortunately use the word "gain," meaning geometric and not arithmetic). It is probably generally accepted by observers experienced in hyperimmunization that the rate of loss of circulating antitoxin decreases with continued immunization, whereas the actual antitoxic titre may change but little. The conception of the "pure" secondary response is new, and Holt (1950*b*) claims that on occasion a "pure" secondary response can be worked out by subtracting the primary base-line titre and the primary response at corresponding times from the total secondary response: this "pure" secondary response, or "leap," is claimed to show a geometric loss in titre with time, that corresponds well with the rate of loss of globulin molecules. Holt states (on p. 234), however,

that it is only from certain types of secondary response curves that the " pure " secondary response can be derived. It is important to know how often such curves are obtained, and whether they have ever been obtained apart from those in which pooled sera or average values of groups have been used.

Our results, given in Table I, show that in the majority of cases both the primary response and its subsequent base-line are negligible in comparison with the total secondary responses. The latter therefore approximate very closely to Holt's " pure " secondary responses. Consideration of the values in Group 5, Table I, shows that some of the rates of loss among individual animals treated in the same manner are quite different, whether the values adopted as the final " base-line " are taken as those recorded for 3 months or for 6 months after the second injection. This is clear from Fig. 7, and the same differences may be seen among individuals in other groups in Table I.

In drawing the conclusions quoted above, Holt (1950*b*) appears to have plotted points obtained by subtracting the values of two sets of pooled sera (one of these the constant " base-line titre ") from that of a third ; it is possible that average values of individuals were used. In one experiment one of the sets of values subtracted was that of an entirely different group of animals (Table III, Series B, p. 236). Two of the four points finally plotted on a straight line from figures obtained as a result of these calculations (Fig. 3, p. 238) were derived from values originally interpolated from a free-hand curve (Fig. 1 and Table II, pp. 235–6). In another experiment a graph was constructed (Fig. 4, p. 239), the points for which were obtained by subtracting the average values of serum from one group of guinea-pigs from the average values for another group at corresponding times (Table IV, p. 239). These animals had received a single injection of P.T.A.P. and the subcutaneous nodules had been excised 7 days and " 14 days or later " after this injection. The difference between these values was taken to represent a secondary response effect superimposed on a pure primary response (see Holt, 1949, p. 292). It was claimed (Holt, 1950*b*) that a linear relation existed between the logs of these differences and time after injection, but it should be noted that the critical point determining the relation was obtained by subtracting 0·6 from 0·7 (Table IV, p. 239). An error of less than 10 per cent in

TABLE I.—*The Antitoxic Values of Individual Guinea-pigs Injected with* 0·25 *Lf Dose of P.T.A.P. at the Time of a Second Similar Injection and at Fixed Times thereafter.*

Group.	Time of reinjection.	A.T. value at reinjection.	A.T. value after 2nd injection (units/ml.).					
			10 days.	1 month.	2 months.	3 months.	4 months.	6 months
1	1 month	0·10	5	2	1	0·5	0·5	0·5
		0·01	5	1	0·5	0·5	0·2	0·2
		0·02	2	2	2	2	1	1
		0·10	2	2	0·5	0·2	0·2	0·2
		0·04	2	1	0·5	0·2	0·2	0·2
		0·002	2	0·5	0·5	0·5	0·5	0·5
		0·02	2	0·5	0·5	0·2	0·2	0·2
		0·01	2	0·5	0·2	0·5	0·5	0·5
		0·004	1	0·5	0·5	0·5	0·5	0·2
		0·10	1	0·5	0·2	0·2	0·2	0·2
		0·002	0·2	0·2	0·2	0·2	0·2	0·2

TABLE I—(*cont.*).

Group.	Time of reinjection.	A.T. value at reinjection.	A.T. value after 2nd injection (units/ml.).					
			10 days.	1 month.	2 months.	3 months.	4 months.	6 months
2	2 months	0·20	10	10	5	2	1	1
		0·02	10	5	5	2	2	1
		0·10	10	5	2	2	1	1
		0·01	5	5	5	2	2	2
		0·04	5	5	5	2	2	2
		0·04	5	5	2	2	2	1
		0·04	5	5	2	2	1	1
		0·10	5	2	2	2	1	0·5
		0·01	5	2	2	1	1	0·5
		0·004	2	1	0·5	0·2	0·2	0·2
3	3 months	0·02	10	10	10	10	5	5
		0·10	10	10	5	2	2	2
		0·004	10	10	5	5	2	1
		0·01	10	5	2	2	2	0·5
		0·001	5	5	2	2	2	1
		0·02	5	5	2	2	2	1
		0·02	5	5	2	2	2	1
		0·02	5	5	2	2	2	0·5
	Under	0·001	5	2	2	1	1	0·2
4	4 months	0·10	20	20	10	10	5	5
		0·10	20	10	10	5	5	2
		0·01	20	10	5	5	2	2
		0·10	20	10	5	5	2	2
		0·04	10	10	5	5	2	2
		0·02	10	10	5	2	2	2
		0·02	10	5	2	2	2	2
	Under	0·001	10	5	2	2	0·5	0·5
	,,	0·001	10	5	2	2	0·5	0·5
		0·004	5	5	5	2	1	1
		0·04	5	2	2	2	1	1
		0·04	2	1	2	2	1	1
	Under	0·001	2	2	1	0·2	0·2	0·5
5	6 months	0·04	10	10	5	5	5	5
		0·10	10	10	5	5	5	5
		0·04	10	10	5	5	5	5
		0·10	10	10	5	2	2	1
		0·04	10	10	5	2	1	1
		0·04	5	5	5	2	2	2
		0·10	5	5	2	2	1	1
		0·04	5	5	2	1	1	1
		0·004	2	1	0·2	0·2	0·2	0·1
	Under	0·001	1	0·5	0·1	0·04	0·04	0·04

TABLE II.—*The Response of Guinea-pigs to First and the Second Injections, each of 0·25 Lf Dose of P.T.A.P.*

Antitoxic value 2 months after 1st injection.	Value 10 days after 2nd injection. 1. 2 units/ml. Number of animals.	5. 10 units/ml. Number of animals.
Under 0·02 unit/ml.	6	3
0·02 to 0·04 unit/ml.	9	8
0·1, 0·2 unit/ml.	1	5

each of these values would have converted the straight line into a definite curve, and the position of the adjacent point in the graph suggests that such a curve would have fitted the points better than the line that was actually drawn.

The suggestion that the secondary response " leap " represents the release of stored antibody is an attractive one. We feel, however, that there is as yet no satisfactory evidence to support it, or to justify Holt's conception of " pure responses " to stimulation. We suggest that a " pure primary response " could more logically be regarded as one that was concerned exclusively with the establishment of good potential immunity ; this can be achieved without any detectable antitoxin appearing in the circulation.

It is difficult to visualize the accumulation and storage of considerable quantities of antitoxin in the body of an animal in whose circulation none has ever been detected before secondary stimulation. In other cases antitoxin may have been lost from the circulation for many years, and yet a typical secondary response may follow an injection of prophylactic. Likewise it is difficult to see why the magnitude of the " leap " from animals primarily stimulated by the same dose of prophylactic should in some circumstances vary according to the type of antigen used as secondary stimulus (our own unpublished observations). Above all, the almost negligible loss of antitoxin from the circulation of some of the guinea-pigs in our experiment, over a period of 6 months, appears to provide strong evidence against the suggestion of the release of stored antibody. This hypothesis cannot account for the considerable differences existing in the rates of loss of antitoxin from the blood of individual animals. Our tests were made at wide intervals, but the individual differences are far beyond the limits of error of the experiment.

The mechanism of antitoxin production in secondary stimulation is obscure, and primary and secondary responses cannot be adequately explained without careful consideration of intermediate response curves. Much work is needed to determine the essential character of potential immunity as established by primary stimulation, and its effect on the subsequent immunological behaviour of animals after further injections.

SUMMARY.

The amount of antitoxin produced following a single injection of prophylactic into guinea-pigs does not necessarily constitute a measure of the degree of potential immunity established. Whereas 1 Lf dose of diphtheria prophylactic P.T.A.P. stimulated greater antitoxin production than the same dose of A.P.T., a remarkable similarity was observed in the immunological behaviour of the guinea-pigs after a second injection of the same prophylactic. This similarity was seen in the heights of the response 10 days after the second injection, and in the rates of loss in

parallel groups that received the second injection 1, 2, 3, or 6 months after the first. Potential immunity (as judged by magnitude of secondary response) increased between the first and second months after primary stimulation and appeared to be maintained thereafter up to 6 months.

When a lower dose (0·25 Lf) of P.T.A.P. was used, potential immunity increased in some but not all animals up to 4 months after the first injection: after 6 months it appeared in general to have declined. The rates of loss of antitoxin in individual guinea-pigs after the second injection showed considerable variation. In general the amount of antitoxin lost by good responders, from 10 days to 6 months after the second injection, decreased as the interval between the injections was increased. Considerable variation was found in the amounts of antitoxin lost by some animals treated in the same manner.

The results obtained using distinguishable guinea-pigs and testing individual sera show the nature of the essential information lost by testing pooled sera. It is suggested that the results obtained by some workers using pooled serum might not have been obtained had individual sera been titrated. Erroneous conclusions concerning fundamental processes may at times have been drawn in consequence.

There is little evidence to support Holt's conception of "pure" primary and "pure" secondary responses, or the suggestion that a secondary response "leap" is due to the release of stored antitoxin.

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SOME NEW OBSERVATIONS ON DIPHTHERIA TOXIN AND ANTITOXIN.

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FOLLOWING the extensive study of Eaton (1936*a*, *b*) on the purification of diphtheria toxin, Pappenheimer (1937) applied fractionation methods to diphtheria toxin which had been prepared on medium of defined composition instead of the customary peptone medium used by Eaton. The purity of the toxins obtained by these two workers, when defined in terms of units of toxin (Lf) per mg. of nitrogen, was of a similar order notwithstanding the difference in the type of medium used. Later, Pappenheimer and Robinson (1937) measured the nitrogen content of the floccule precipitate produced from the interaction of a constant dose of antitoxin (300 units) with varying amounts of toxin. For certain ratios of antitoxin to toxin, after removing the floccules by centrifuging, they found the supernatants to be devoid of either free antitoxin or free toxin by the tests which they used. This range they defined as the equivalence zone, and assuming that in this zone all the added antitoxin and toxin appeared in the washed precipitates, they deduced values for the nitrogen content of antitoxin (0·0016 mg. N per unit) and toxin (0·00046 mg. per Lf). This was made possible by the fact that for the toxin and antitoxin used by them the nitrogen values for the floccules in the equivalence zone were on a straight line which could be extrapolated to zero toxin, thus giving the nitrogen value for 300 units of antitoxin. The nitrogen value for the toxin could be obtained from the slope of this linear portion of the curve (see Fig. 1). The value so obtained for the nitrogen, associated with 1 Lf unit of toxin was in close agreement with the value for toxin purified by Eaton and also by Pappenheimer, and it appeared that this method, which depended on the specific precipitation of the antitoxin by toxin, gave final proof to the view that these isolated products were in fact virtually pure toxin for which a value of 0·00046 mg. N/Lf or 2170 Lf per mg. N had now been established. Pappenheimer and Robinson state that the values which they obtained were independent of the purity of the toxin used to produce the floccules, and moreover, claimed that by the use of their method, the value of "unknown strength" antitoxins and toxins in terms of units could be determined in the absence of "standard antitoxin."

The value obtained by Pappenheimer and Robinson for the purity of diphtheria toxin has been widely accepted, and it appeared that, while there was still no certain knowledge of the mechanism by which the toxin exerted its toxic action, at least we knew its molecular weight, and had some idea of its chemical composition (Pappenheimer, 1937; Pappenheimer, Lundgren and Williams, 1940).

Observations made in these laboratories during the past year have brought to light so many facts which are not in line with previously held views that we wish in this paper to give a short account of the work already done, although it is realized that this is as yet incomplete. A more detailed account of the work will be given elsewhere.

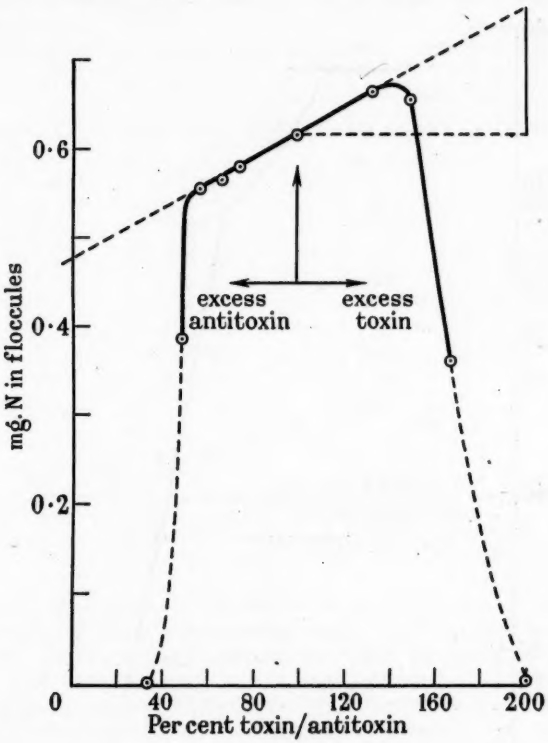


FIG. 1.

Derrien (1947) employed 3·5 M potassium phosphate as a protein precipitant and found that, provided the increments of 3·5 M phosphate were sufficiently close and sufficient time was allowed for equilibrium to be reached, the precipitation of human serum showed a large number of exponential steps which indicated the presence of many proteins with different solubilities.* This method shows some advantages over the use of ammonium sulphate for the fractionation of the proteins present in the crude toxic filtrates of *C. diphtheriae*, and we have applied it with considerable success in our work. After trial of phosphate mixtures of varying pH values we selected one of pH 7·4. The crude culture filtrates were first concentrated by ultrafiltration, and as much non-protein nitrogen as possible

* For an exponential curve a plot of log soluble toxin (or protein) against percentage concentration of precipitant (or ionic strength) gives values which fall on a straight line.

was removed by washing on the ultrafilter with 1/10,000 thiomersalate saline. In the fractional precipitation experiments the amount of crude toxin and the final volume were constant and only the amount of 3·5 M phosphate varied. After allowing a period of 24 hours for equilibrium to be reached, the mixture. were filtered and the first portion of filtrate discarded. Examination of the

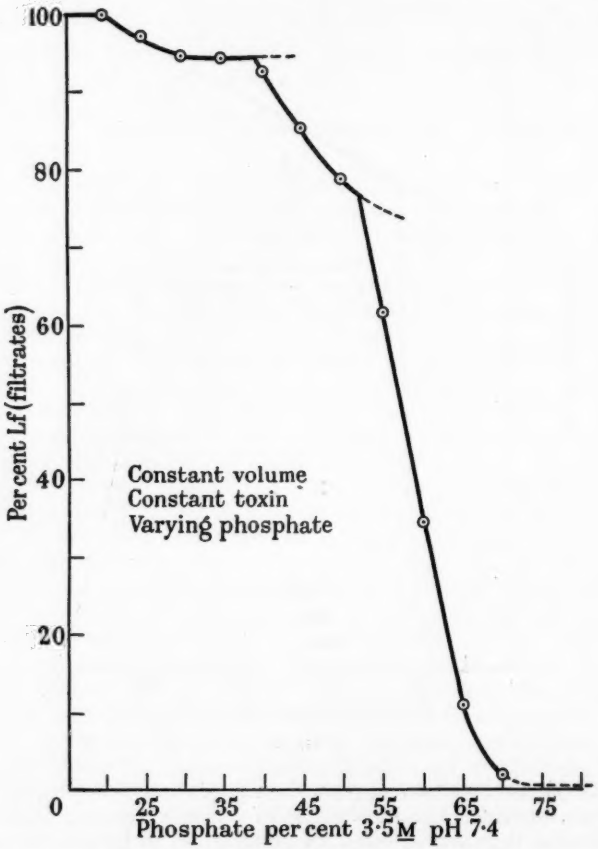


FIG. 2.

filtrate for its protein precipitation (using a Beckman Spectrophotometer at 280 mμ.) or for toxin (Lf determination) showed that precipitation had taken place in a series of exponential steps; the steps were at identical phosphate concentrations, whether measured from protein or Lf precipitation. Different batches of toxin or toxoid showed variation in the number of exponential steps which could be detected in this way, but up to eleven have been found. Fig. 2 is an example of the type of result obtained by this method. The toxin, as determined

by flocculation tests, was not associated with the proteins of any one of these exponential curves but was associated with all the proteins present. Further work in which toxin was separated into a series of fractions by means of phosphate precipitations showed that the fractions differed in their properties as measured by the number of Lf units per mg. of nitrogen and in their Kf value (time of flocculation with antitoxin). Re-precipitation of these fractions did not materially affect their properties.

Table I illustrates the fractionation of a batch of diphtheria toxoid by this method.

TABLE I.—*Fractionation of Diphtheria Toxoid by Phosphate.*

Proportion of 3·5 M phosphate added.	Yield Lf per cent.	Units/mg. protein N.	Kf 30*.
0–30%	1·4	424	30 min.
30–37%	3·6	240	1–2 days
37–43%	5·3	343	7 hours
43–46%	6·7	603	85 min.
46–50%	11·5	815	40 ,,
50–54%	17·0	1170	35 ,,
54–58%	16·3	1370	22 ,,
58–62%	12·5	1320	30 ,,
62% filtrate	7·8	—	—
	82†		

* Kf 30 : Time required for first flocculation of a mixture ; toxoid used at 30 units/ml. (1·0 ml.) diluted to 2·0 ml. in test. Antitoxin = peptic refined antitoxin. Bath temperature 40°.

† Each precipitate was washed with a little phosphate-water mixture at the appropriate concentration and the Lf value of these washings is not included in the total yield.

These results are typical of those obtained for toxins or toxoids and the results are much the same whether the toxin has been obtained from tryptic digest medium, papain digest medium or casein hydrolysate medium. Both Eaton (1936) and Pappenheimer (1937) have noted the presence of low-purity slow-flocculating material in early ammonium sulphate fractions, but they do not appear to have studied these fractions to any extent.

We were interested to know whether these low-purity slow-flocculating fractions were produced by the adsorption of toxin on to other proteins or to loose combination of toxin with other proteins. We considered that the method of Pappenheimer and Robinson (1937) which involved the formation of specific floccules and the determination of the floccule nitrogen might throw light on this point, since they had found the floccule nitrogen value to be independent of the purity of the toxin used.

In a very large number of experiments which we have carried out, using crude toxins produced on different types of medium, and fractions prepared from them of widely varying purities in terms of Lf units per mg. nitrogen, we have failed to obtain a single curve of the type reported by Pappenheimer and Robinson (1937) or Cohn and Pappenheimer (1949) for horse antitoxin. The antitoxin used has been either ammonium sulphate-fractionated material, or pepsin-refined antitoxin (Pope, 1938, 1939) produced at these laboratories, or

antitoxin obtained from other sources, including antitoxin from America, Germany and this country. Fig. 3 shows a typical result for three toxins against the same peptic-antitoxin. Two of the toxins were crude products which had been concentrated and washed on an ultrafilter; the other was a highly purified fraction with a purity of 2830 Lf/mg. P.N. obtained by phosphate fractionation. It will be

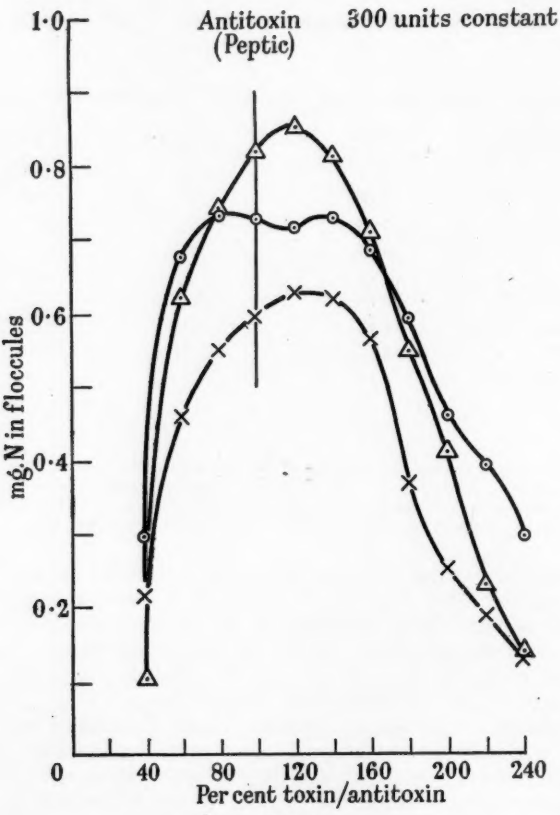


FIG. 3.

seen that each of these toxins gave a different curve for the nitrogen content of the floccules produced, and that at the neutral point the highly purified toxin gave the lowest figure. However, it must be pointed out that the value for this purified fraction (2830 Lf/mg. P.N.) is greater than the value (2170 Lf/mg. N) deduced by Pappenheimer and Robinson (1937) from floccule nitrogen measurements.

At this stage we were unable to say whether the difference between our results was due to the toxin or the antitoxin used. We had available some

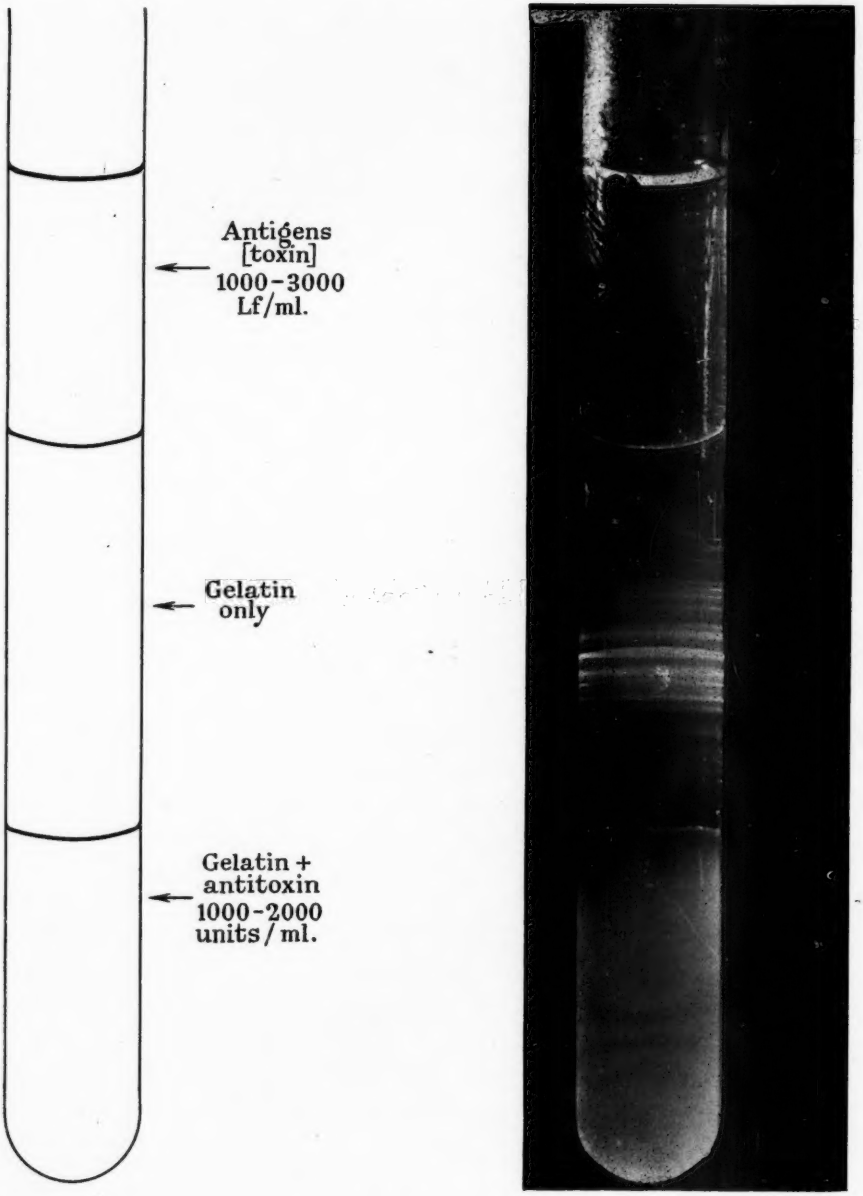


Fig. 4.

Pope, Stevens, Caspary and Fenton.

purified toxin which had been sent by Dr. A. M. Pappenheimer, Jr., to one of us (C. G. P.). This product was a freeze-dried preparation stated to contain 0·00046 mg. N per Lf unit (2170 Lf/mg. N). After solution and assay by the flocculation method, neutral floccules were prepared exactly as described by Pappenheimer and Robinson (1937). Three antitoxins were used: a routine peptic-antitoxin, a peptic-antitoxin from the Lister Institute, and flocculeantitoxin prepared as described by Pope and Healey (1939) by the action of pepsin on washed neutral floccules. The figures for the nitrogen values found per toxin-antitoxin unit [TA] for the three antitoxins used are shown in Table II, where they are compared with the value expected for peptic antitoxin from the work of Pappenheimer and Robinson.

TABLE II.—*The Results obtained for the Nitrogen per [TA] Unit with Pappenheimer's Toxin and Three Peptic Antitoxins.*

Antitoxin used.	N per [TA] unit.
W.R.L. Peptic . . .	$22{\cdot}3 \times 10^{-4}$ mg.
Lister Institute Peptic . .	$23{\cdot}7 \times 10^{-4}$ mg.
"Floccule" antitoxin . .	$21{\cdot}3 \times 10^{-4}$ mg.
Pappenheimer and Robinson value .	$16{\cdot}1 \times 10^{-4}$ mg.

It is clear from these results that even the use of Pappenheimer's purified toxin, having a value of 2170 Lf/mg. N, does not yield with the peptic antitoxins used by us figures for the nitrogen content of the floccules which agree with those reported by Pappenheimer and Robinson. It might be argued that our antitoxins contained antibodies other than antitoxin and that these were being precipitated in the floccules, but this would also imply that Pappenheimer's highly purified toxin contained other antigens which could cause their precipitation. Pappenheimer states that his highly purified toxin does contain a small amount of bacterial proteins, but considers that this contamination does not exceed about 5 per cent (Cohn and Pappenheimer, 1949).

In view of these conflicting results we turned to another method which we hoped would throw some light on the problem. Oudin (1946, 1947, 1948*a*, *b*, 1949), Elek (1948), Ouchterlony (1948) and Munoz and Becker (1950) have used methods in which toxin and antitoxin (or other antigen-antibody systems) are allowed to diffuse in agar-gel to produce precipitin bands or lines. The method which we have used is based on that of Oakley (unpublished). We prefer the use of gelatin instead of agar and allow the lines to develop in the cold room at about 3°. Fig. 4 shows the method used and a typical result obtained by using peptic antitoxin and a toxin purified to 1630 Lf/mg. N; at the time it was photographed there were 14 lines. In order to demonstrate the maximum number of antigen-antibody systems it is essential that the concentration of antitoxin (and other antibodies) in the bottom layer of gelatin shall be high, and we like to have at least 2000 flocculating units per ml.; it is also an advantage to use antibodies which have been purified by peptic digestion because the smaller molecules diffuse more rapidly. The antigen system should also be concentrated to approximately 1000 Lf units per ml. In our opinion this method has proved to be invaluable as a means of elucidating the antigenic structure of diphtheria toxin-filtrates and as a guide to the purification of both toxin and antitoxin.

Using this method we have detected up to 24 antigens in crude culture filtrates from *C. diphtheriae* (PW 8 strain), and this number must be regarded as a minimum and not a maximum. When we applied this test to the purified toxin obtained from Dr. Pappenheimer we were able to show the presence of 14 antigens, of which 7 were major and 7 minor in terms of the density of the lines produced. An examination of our own purified toxins showed that they also gave a similar number of antigen-antibody lines. However, by the use of new purification methods, and with this test as a guide, we have obtained purified toxin which gives only 3 lines against the test antitoxin.

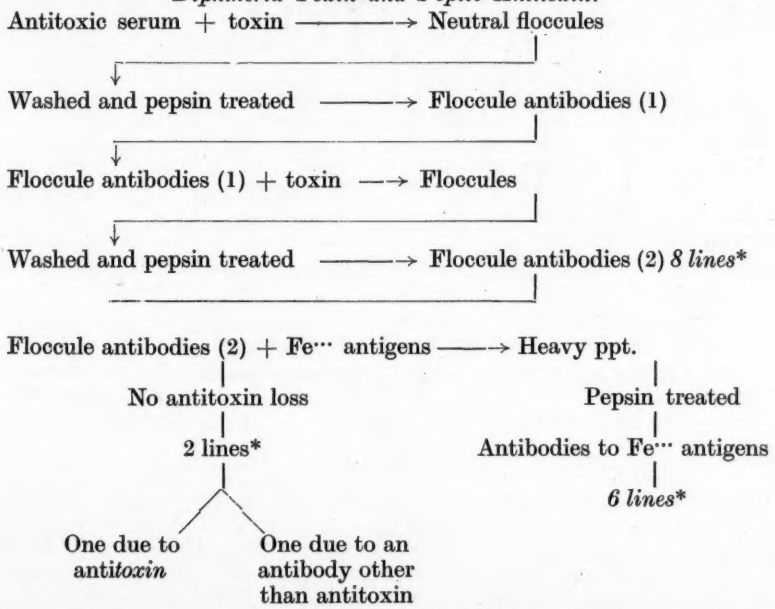
The examination of antitoxin obtained from various sources has also shown variation in the antibody content; in general it may be said that antitoxin of English and German origin shows the presence of a larger number of antibodies than do various samples of antitoxin obtained from the U.S.A. We have some evidence that this difference is related to the methods of hyperimmunization used; in the U.S.A. a relatively short immunization is used whereas, at least in England, the horse immunization is continued over a much longer period. The longer period would allow the development of antibodies to the weaker antigens present in the crude toxin or toxoid employed for immunization. From the examination of a number of samples of American antitoxin we have found the number of detectable antibodies to be about half the number found in English samples, but in no case have we found only a single antibody (i.e., antitoxin) to be present.

With the knowledge that even highly purified toxin fractions contain a number of different antigens, and that the antitoxic sera contain a number of antibodies, it is necessary to examine the term "specific precipitation" as applied to the toxin-antitoxin floccules, and to determine whether these floccules do in fact contain toxin and antitoxin only or whether they include other antigen-antibody precipitates. We have prepared a number of batches of neutral floccules from different types of diphtheria toxins and antitoxins, and these, after washing with saline, were treated with pepsin according to the method of Pope and Healey (1939) to recover the antibodies from the floccules. These "floccule-antitoxins" were then tested by the diffusion method; in every case they showed the presence of several antibodies, varying in number from 6 to 12, depending on the toxin or antitoxin used.

In Table III we show the results for a typical experiment of this type, in which the antitoxin (with other antibodies) recovered from the neutral floccules by pepsin treatment was re-precipitated a second time with toxin and then recovered once more from the second batch of floccules. This was done to ensure that antibodies, other than antitoxin, were not being carried over from the original antitoxin used, notwithstanding the fact that the floccules had been well washed with saline. However, the recovered "floccule-antitoxin" still showed the presence of 8 antibodies in the diffusion test. When *C. diphtheriae* is grown in the presence of an excess of iron, toxin production is inhibited (Cohn and Pappenheimer, 1949). A culture filtrate prepared in this way was concentrated by ultrafiltration and then examined for its toxin content. It had an Lf value of < 1 unit per ml. (blend test [Glenny and Okell, 1924]), whereas a typical toxic filtrate concentrated to the same degree would have an Lf value of 1000–2000 units per ml. This material ($Fe^{\cdots}$ antigens) showed a number of diffusion lines; it also gave a heavy flocculation with floccule-antitoxin (2). The optimal ratio

was determined and the required amount of $Fe^{\cdots}$ antigens was added to floccule-antitoxin (2); a very heavy precipitate formed rapidly; this was allowed to settle and was later removed by centrifuging. No loss of antitoxin could be detected in the supernatant, which retained its original flocculating value (corrected for dilution with $Fe^{\cdots}$ antigen). The precipitate, treated with pepsin, yielded antibodies which gave 6 lines when tested against the $Fe^{\cdots}$ Antigen System, but no antitoxin was found in this fraction (blend test with standard antitoxin). On the other hand, the adsorbed floccule-antitoxin now showed only two lines when tested against crude toxin. Of these two lines, one was shown to be due to an antigen other than toxin by absorbing 1000 Lf/ml. crude toxin with Fuller's earth until the Lf value had been reduced to zero. The adsorbed filtrate still produced one of these lines, and contained therefore an antigen other than toxin.

TABLE III.—*The Antigen-Antibody Composition of the Floccule Precipitate from Diphtheria Toxin and Peptic Antitoxin.*


Antitoxic serum + toxin → Neutral floccules
Washed and pepsin treated → Floccule antibodies (1)
Floccule antibodies (1) + toxin → Floccules
Washed and pepsin treated → Floccule antibodies (2) 8 lines*
Floccule antibodies (2) + Fe··· antigens → Heavy ppt.
No antitoxin loss
2 lines*
One due to antitoxin
One due to an antibody other than antitoxin
Pepsin treated
Antibodies to Fe··· antigens
6 lines*

* Shows the number of antigen-antibody lines obtained in the diffusion test against crude ultrafiltered toxin.

It will be seen that when the antigen system was toxin-free, the production of a very heavy precipitate of other antigen-antibodies did not lead to the inclusion or co-precipitation of antitoxin, and it is therefore improbable that the precipitation of "pure toxin" with antitoxin would lead to the inclusion of other antibodies in the precipitate. On the other hand, the presence of other antigens in a purified preparation would give contaminated floccules, provided the antitoxin used contained antibodies to these antigens. We have purified toxin to a

value equal to that obtained by Pappenheimer (1937) and used it to prepare floccule-antitoxin. The recovered antitoxin tested at a high unit per ml. value by the diffusion test showed the presence of 12 antibodies. We regard this as further evidence that toxin with a purity of the order of 2170 Lf units per mg. N is not a pure material; in fact we consider the amount of contaminating material must be substantial, and our most recent work has done nothing to modify this view.

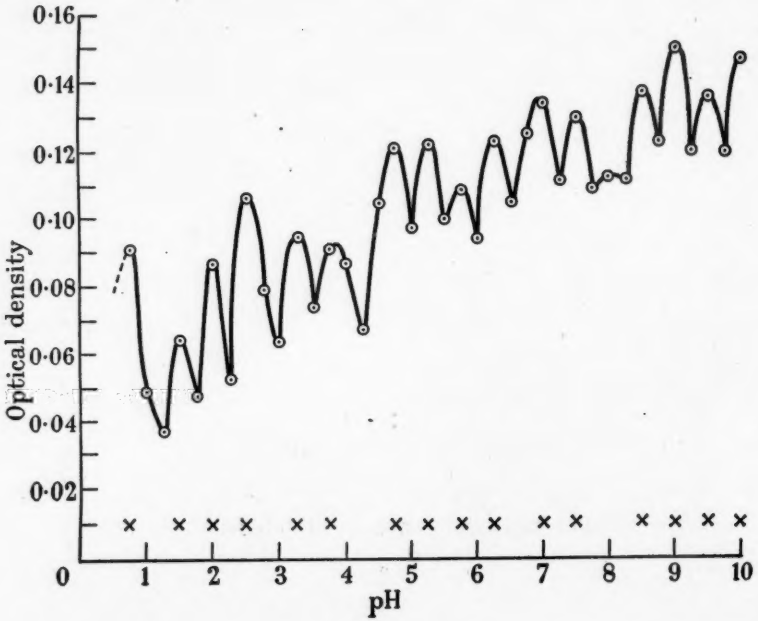


FIG. 5.

With the knowledge that crude toxin filtrates contained a very large number of antigens capable of producing corresponding antibodies in the hyper-immunized horse we have endeavoured to obtain more information about these antigens. In connection with other work we have developed a very sensitive method based on the work of Anson (1938) for the determination of proteolytic enzyme activity across the pH range 0·75–10·0 in steps of 0·25 pH unit. To our surprise we found that the application of this method revealed the presence of a large number of proteolytic enzymes in the culture filtrates from *C. diphtheriae*. These proteolytic enzymes were present in culture filtrates from all sub-strains of P.W. 8 and all *C. diphtheriae gravis* examined, but the *gravis* strains appear to produce higher concentrations of enzymes active at alkaline pH values. The quantity of enzymes present in the culture filtrate bears some relation to the type of medium used; filtrates from tryptic digest medium, containing originally proteoses and polypeptides, yield larger amounts of these enzymes than do

filtrates from casein-hydrolysate medium, where the casein has been broken down to amino-acids by acid hydrolysis. These enzymes are thermolabile, but there is considerable difference in the temperatures at which they are inactivated. They are also produced in culture filtrates where an excess of iron has been added to suppress toxin formation. An examination of toxin that has been highly purified shows that while some of these enzymes have been eliminated almost completely, others have been concentrated with the toxin in the purification process.

TABLE IV.

Toxin : RX 4112 *gravis* strain CN 2026.
Substrate : Crystalline horse haemoglobin.
Antitoxin : From a horse immunized with *C. diphtheriae gravis* Dublin 9 strain toxoid.
Digestion time 6 hours at 40°. Sampled at 0·5, 1·0, 2·0, 4·0 and 6·0 hours.

Enzyme with peak activity at pH.	Inhibition of enzyme activity by anti-serum.		
	1 equivalent.*	2 equivalents.	3 equivalents.
0·75	Inhibited	—	—
1·5	,,	—	—
2·0	Nearly inhibited	—	—
2·5†	Slightly inhibited	Partially inhibited	Partially inhibited
3·25	,, ,,	,, ,,	Almost inhibited
3·75	No inhibition	Inhibited	—
4·75	Almost inhibited	—	—
5·25	Inhibited	—	—
5·75	,,	—	—
6·25	Partially inhibited	Partially inhibited	Inhibited
7·0	,, ,,	,, ,,	Almost inhibited
7·5	Slightly inhibited	,, ,,	,, ,,
8·5	Partially inhibited	Inhibited	—
9·0	Almost inhibited	,,	—
9·5	,, ,,	—	—
10·0	,, ,,	—	—

* 1 equivalent of antitoxin contains the same number of antitoxic units as the number of Lf units present in the dose of toxin used.
† 3 equivalents had about the same effect as 2 equivalents.

We have been able to show that the activity of these enzymes is inhibited by antibodies present in the serum of hyper-immunized horses. Fig. 5 shows the enzymes present in a culture filtrate from *C. diphtheriae gravis* (CN 2026), determined by their action on crystalline horse haemoglobin, used as substrate, at varying pH values. Table IV shows the capacity of *gravis* antitoxin (produced by immunizing a horse with *C. diphtheriae gravis* Dublin 9 strain) to neutralize these enzymes.* Many of the enzymes were neutralized by a dose of antitoxin equivalent to the Lf value of the toxin ; others required two or more equivalents of antitoxin. When neutralized in this way they no longer showed any proteolytic activity against haemoglobin. It is of interest here to point out the enzyme

* The action of *C. diphtheriae* P.W. 8 antitoxin on the proteolytic enzymes of *C. diphtheriae gravis* is being examined.

which had peak activity at pH 2·5; for this enzyme there appeared to be little or no corresponding antibody in the antitoxin used, and we think this indicates that strain CN 2026 produced an enzyme which was not present in more than minute quantity in the filtrate from *C. diphtheriae gravis* Dublin 9 which was used to immunize the horse.

The presence of these proteolytic enzymes, which we have shown to be antigens in that they produce antibodies in the immunized horse, would account for about 15 out of 24 antigen-antibody systems which we have found. We think it probable that other non-proteolytic enzymes are also present in the culture filtrate, but so far we have not investigated this point.

Another type of antigen or hapten system has been obtained by applying the method of Raistrick and Topley (1934) to the washed *C. diphtheriae* obtained from the routine production of toxin. *C. diphtheriae* P.W. 8 strain yields after tryptic digestion a protein-free filtrate which contains a material precipitable by alcohol; this fraction precipitates with peptic antitoxin (P.W. 8), giving a typical flocculation reaction, and our recent results indicate that at least three antigenic residues are present in this material. A similar product obtained from *C. diphtheriae mitis* shows a very much weaker reaction against peptic antitoxin (P.W. 8), and that from one strain of *C. diphtheriae gravis* shows none against P.W. 8 antitoxin. These fractions are probably related to the somatic antigens, but it is of interest that culture filtrates derived from *C. diphtheriae* P.W. 8 must have contained these antigens (in less degraded form), as shown by the presence of corresponding antibodies in the P.W. 8 antitoxin. Relatively small quantities of these materials are obtained from 100 g. amounts of *C. diphtheriae* and we have not yet been able to investigate their chemical properties; it is probable that they are, in part, polysaccharide in structure.

The shape of many of the floccule nitrogen curves, of the type shown in Fig. 3, which we have obtained, suggested very strongly that we were dealing with a compound flocculation in which many antigens and antibodies were involved, and that in consequence the Lf value of a toxin (or the flocculation value of an antitoxin) might differ from that which would be obtained if a single antigen (toxin) were reacting with its specific antibody (antitoxin). This would be in keeping with the work of Taylor and Adair (1935), who showed that two distinct antigen-antibody systems could be brought nearer each other until a point was reached when they suddenly fused together to give a single flocculation.

A more detailed examination of the major diffusion line, using a slightly modified technique, revealed the fact that it was compound and could be resolved into at least three components, each line being about equal in density. This fact implies that these antigens must be present in substantially equal quantities (almost equal diffusion rates), and the same applies to their corresponding antibodies. Assuming therefore that the usual Lf flocculation system between toxin and antitoxin is composed in the main of two or three major antigens and their antibodies (neglecting the minor antigen-antibody systems which we have shown to be involved), it follows that a method which would cause progressive destruction of one of these antigens should lead to the production of a double flocculation system. That is to say, the original flocculation should remain at practically unaltered value, and a second flocculation corresponding to the residual value of the antigen undergoing destruction (provided the destruction is not complete) should appear.

Further work on this point has yielded three methods by means of which virtually complete destruction of the specific toxin can be achieved without very marked effect on the flocculation value. Two of these methods involve the use of proteolytic enzymes which destroy the specific toxin very rapidly while having much less action on the other antigens present. The third method depends on the destruction of the specific toxin with *n*-butyl alcohol, again with little effect on the other antigens or the Lf value. For example, if crude casein hydrolysate toxin, concentrated by ultrafiltration to 2000 Lf per ml., is acted upon by trypsin at room temperature, and samples are tested at intervals, it is found that one Lf value is obtained which corresponds to >80 per cent of the original; when sufficient of the specific toxin has been destroyed a second flocculating zone appears, and the value of this falls steadily with toxin destruction. A plot of these two Lf values against the time of digestion shows that both have originated from the original Lf value. The antigen(s) responsible for the flocculation at >80 per cent of the original value are relatively stable to the action of trypsin whereas the specific toxin is rapidly destroyed. The fact that the antigen undergoing destruction was specific toxin was checked by blend tests (Glenny and Okell, 1924).

When these methods were applied to a toxin purified to a value of 2290 Lf per mg. N, they showed quite clearly that here also the specific toxin could be virtually destroyed, leaving a flocculating system which did not depend for its apparent Lf value on a toxin-antitoxin system, but on other antigens and their antibodies. The floccules of this system contain little or no antitoxin, and practically all the true antitoxin (except for the small amount neutralized by the residual trace of toxin) remains in the supernatant. The presence of these antigens constitutes definite proof that this material is not pure toxin.

We consider that by three distinct methods, (*a*) the nitrogen content of the floccules, (*b*) the diffusion line method and (*c*) destruction of the specific toxin leaving other antigens which still flocculate, we have shown that the isolation of pure diphtheria toxin has not yet been achieved. Using new fractionation methods we have obtained small yields of material with a value of 3200 Lf/mg. N, but we do not consider this to be pure toxin.

The presence of a large number of antigenic substances in culture filtrates from *C. diphtheriae* will naturally raise the question of their significance in infection. We cannot answer this question, but we would draw attention to the present tendency to employ highly purified toxoid in the production of prophylactics. Assuming that pure toxin (or toxoid) could be isolated, would a prophylactic prepared from it, which would give rise to a pure antitoxin response, provide all the immunity necessary ?

SUMMARY.

The antigenic structure of culture filtrates from *C. diphtheriae* has been shown to be very complex. Toxic filtrates from *C. diphtheriae* P.W. 8 strain contain at least 24 antigens and routine peptic-refined antitoxins contain corresponding antibodies. A large number of these antigens have been shown to be proteolytic enzymes whose activity is neutralized by antibodies present in commercial antitoxins. The amount of antitoxic serum required for neutralization varies with each enzyme. Park-Williams 8 antitoxin also contains three antibodies

which react with hapten residues obtained by digestion of *C. diphtheriae* P.W 8 with trypsin; hapten residues from *C. diphtheriae mitis* react weakly, while those from *C. diphtheriae gravis* do not react with this antitoxin.

The specific floccules resulting from the interaction of toxin and antitoxin do not consist of toxin and antitoxin only; other antigens and antibodies are also precipitated. Deductions made from the nitrogen content of these floccules are of little value; they do not indicate the purity of either toxin or antitoxin.

In the Ramon flocculation test (Lf) the simultaneous precipitation of antigens and antibodies may cause distortion of the true value (for toxin and antitoxin). By selective destruction of the antigens present in either crude or highly purified toxin this compound nature of the flocculation reaction has been demonstrated. Our observations have shown quite clearly that diphtheria toxin has not yet been obtained as a pure substance.

One of us (C. G. P.) wishes to thank Dr. A. M. Pappenheimer, Jr., for a sample of his purified toxin, Dr. J. A. McComb (Boston) for samples of antitoxin, and Dr. W. Maycock (Lister Institute) for a supply of pepsin-refined antitoxin.

The *gravis* antitoxin used was prepared by Mr. A. T. Glenny, F.R.S., and our thanks are due to him for this material; also to Dr. C. L. Oakley for details of his diffusion method.

We also wish to thank Dr. F. V. Linggood and the staff of the toxin laboratory for assistance in producing large amounts of culture filtrates and ultrafiltered toxins used in this work.

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THE ENVELOPE SUBSTANCE OF *PASTEURELLA PESTIS*.

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WHEN it is growing freely in the tissues of susceptible animals or in a favourable culture medium maintained at 37°, the plague bacillus produces a sticky covering substance, the so-called envelope. This structure was first described by Rowland (1912) in one of his reports to the British Commission on Plague in India. The presence of a capsule had been described by Kitasato (1894) and others at a much earlier date, and it is still customary, though probably incorrect, to describe the two as separate entities. The importance of the envelope as a complete antigen was established by the work of Schütze (1932, 1934). Bhatnagar (1940) has confirmed and extended Schütze's findings. The immunology of plague has also been studied extensively by Meyer and his associates in California (see Meyer, 1948, 1950). Recently these workers have shown that aqueous extracts of acetone-dried *Pasteurella pestis* contain a protein-polysaccharide complex which they believe to be identical with the envelope antigen of Schütze (Baker, Sommer, Foster, Meyer and Meyer, 1947 ; Meyer, 1950). The trend of research, therefore, has been to bring *Past. pestis* into line with other capsulated bacteria, the evidence strongly suggesting that the envelope is no more than a particularly well-developed capsule which functions as a complete antigen. It must nevertheless be borne in mind that this identification of the envelope with the protective antigen has not been fully proved. It might, for instance, be argued that the envelope is an inert bacterial slime which overlies the surface antigen. The properties of the envelope can only be exactly determined by separating it completely from the bacterial cell and then submitting it to immunochemical analysis. The purpose of the present study was to find a means of doing this.

The envelope has some claims to be regarded as a morphological entity ; its removal should therefore be capable of demonstration by visual methods. It will be shown here that solution of the envelope by appropriate solvents can, in fact, be observed microscopically. This technique, which will be described in detail, has provided valuable information which would not have been available if the usual methods of immunochemistry had alone been employed. Partial purification of the dissolved envelope material has been achieved but its full chemical characterization requires further investigation. The evidence so far obtained indicates that it is a protein which cannot be dissociated without loss of antigenic properties. The purified material is non-toxic and is effective in high dilution, a few micrograms being sufficient to produce a high degree of immunity in experimental animals. Since the same surface antigen occurs in all strains of *Past. pestis*, and since the purified antigen can be readily obtained from a non-virulent

strain by the methods here described, the possibility of using it for human prophylaxis deserves serious consideration. The antigen is probably very similar to the antigenic Fraction Ia prepared by Baker and his associates. This has already been tested in human volunteers by Meyer and Foster (1948) with very satisfactory results.

EXPERIMENTAL.

Materials and Methods.

It was decided at the outset to avoid all chemical and physical methods which might produce degradation of the envelope and the formation of haptenes. Most of the work was accordingly done with the non-virulent strain Tjiwidej, which could be handled safely without a preliminary treatment with heat or antiseptics to kill the cells. This strain was originally isolated by Otten (1941), who employed it for the production of his living avirulent plague vaccine. It is best maintained on nutrient agar containing 5–10 per cent of serum. Blood agar should not be used, since this favours the production of non-capsulated variants. Unless the medium is well buffered glucose also favours the formation of these variants, probably as the result of acid formation during the early stages of growth. For fluid cultures a buffered broth is recommended. This is prepared by adding to each litre of infusion broth 6·8 g. of KH_2PO_4, and sufficient N/10 NaOH to bring the pH to 7·4. A peptic digest of blood (Fildes, 1920) was added to the finished medium in a concentration of 0·2 per cent. Phenol red was included in all transparent media in a concentration of 0·002 per cent in order to indicate the reaction of the medium during cultivation. With fluid media continuous agitation during growth improved the yield of organisms, but cultivation in an atmosphere containing CO_2 had an unfavourable effect, as was to be expected with an acid-sensitive organism. For large-scale production stainless steel trays with loosely fitting lids were used instead of culture bottles. Each tray provided an agar surface of 1900 sq. cm. and yielded about 7·5 g. of moist bacterial growth. The medium employed was beef heart tryptic digest agar containing 0·05 per cent of glucose. Each tray required 1200 ml. of medium to give a satisfactory depth. Cultivation was carried out for 24–48 hours at 37° in a humid atmosphere. The growth was then gently scraped off the agar, suspended in physiological saline and filtered through muslin in order to remove any pieces of agar. In the case of fluid cultures the medium was dispensed in litre bottles. After 48 hours' incubation the cells were collected by means of a Sharples centrifuge, washed once in the centrifuge and finally suspended in saline.

Experiments on animals.

The immunogenic value of the various antigenic fractions was determined in the usual manner by immunizing animals, and subsequently challenging them with a virulent strain of *Past. pestis*. These experiments were carried out almost exclusively on multimammate mice, *Rattus* (*Mastomys*) *natalensis*. The advantages of using this animal for plague investigation have been described in a recent report from this laboratory (see Report, 1941). This animal plays an important part in the ecology of plague in South Africa. It is highly and uniformly susceptible to infection, about 5 bacterial cells being sufficient to produce a rapidly fatal septicaemia. It breeds readily in captivity, and is relatively free from spontaneous disease. The only disadvantages of these animals are that

they are difficult to handle, being very active and vicious biters; and that separate cages must be used for each animal under test since the sick members of a group are killed and eaten by their cage-mates. In order to obtain significant results groups of 10 or more animals were immunized with each dilution of the antigen, so that a single experiment required 50 to 100 animals. To use guinea-pigs on this scale was impracticable, and the strain of white mice available in this laboratory is much less uniform in its susceptibility to infection. During these investigations more than 2000 *Mastomys* were employed for protection experiments of this kind.

The Demonstration of the Envelope by Microscopy.

With *Past. pestis* the usual methods of staining to show the presence of capsules give disappointing results. The envelope may, however, be easily studied under the microscope in wet Indian ink preparations illuminated by a dark field condenser. This method, at first believed to be original, was in fact used many years ago by Rowland (1912), who succeeded in obtaining some excellent photomicrographs to illustrate his paper. In the present studies this technique was invaluable as a means of following under the microscope the process of solution of the envelope by the various reagents employed. Examined in this manner, the envelope appears as an empty space between the bacterial cell and the background, which is formed by a thick carpet of refractile carbon particles in active Brownian movement. At the somewhat poorly defined interface these particles can be seen bombarding the envelope, but they do not penetrate it. The shape of the envelope can be distorted by gently pressing a needle against the surface of the cover glass and its plastic nature may thus be demonstrated. When the organism has been growing under favourable conditions the width of the envelope may exceed that of the cell itself and several cells may be seen embedded in a single mass of envelope substance.

The technique will now be described in detail. One small loopful of a fairly dense suspension of the organism is mixed on a clean grease-free cover glass with an equal volume of Indian ink. Merthiolate may be added to the stock bottle of Indian ink in a concentration of 1 in 1000 to inhibit the growth of bacterial contaminants. A microscope slide is then gently lowered on to the drop, taking care to avoid the formation of air bubbles. The cover glass is then pressed down firmly until only a very thin film remains, so thin, indeed, that the bacterial cells are almost touching both slide and cover glass. The ink then surrounds the cells on all sides, but does not lie above or below them. The excess of fluid is then removed by touching with a strip of filter-paper. The correct thickness is obtained when the preparation, held up to the light, has the colour of a weak neutral filter of about 90 per cent transmission. In order to prevent evaporation with consequent streaming of the fluid the edges of the cover-glass must be sealed with paraffin or immersion oil. The preparation is then examined by dark-field microscopy in the usual manner. The best results are obtained with an oil-immersion objective fitted with an iris diaphragm so that the effective numerical aperture can be varied during observation. Rowland's method of using an ordinary condenser with a patch stop did not give such good results as a modern bi-reflecting dark-field condenser (Zeiss cardioid). Some typical appearances are shown in the photomicrographs reproduced in Figs. 1–6. It

will be noted that the pneumococcus, *Klebsiella pneumoniae* and *Bacillus mycoides* when examined by this method show " envelopes " which are as well developed as those of *Past. pestis*.

For the success of this work it was essential to maintain the strains employed in a phase in which envelope production was at a maximum. Here again the Indian ink technique was of great value, since it provided a means of estimating the amount of envelope material present and of detecting the appearance of non-capsulated variants. Repeated subcultivation eventually gave rise to dissociative changes irrespective of the type of culture medium used. The change to the non-capsulated type was heralded by the appearance of large ovoid cells with a strong affinity for fuchsin. These first formed a minority, but rapidly became the predominant cell type on further subcultivation. They were easily recognized in the ink preparations, and were entirely lacking in envelope substance. The photomicrograph reproduced in Fig. 3 shows a number of these cells, together with others that vary considerably in the extent to which envelopes are present. Plate cultures revealed several of the abnormal colonial types described by Bhatnagar (1940). This variant was isolated and proved stable on subculture. It retained the biochemical characters of *Past. pestis*, and stimulated the production of somatic antibody in rabbits after a long course of injections. The rabbit antisera had no protective action against a virulent strain, but gave a precipitin reaction with antigens prepared from the homologous strain and from normal *Past. pestis*. All attempts to restore the strain to normal were fruitless, and the only expedient was to return to a seed culture preserved at a time when the strain was behaving normally.

Separation and Purification of the Envelope Substance.

The capsular polysaccharides of the pneumococcus readily dissociate from the bacterial cell to form colloidal solutions in the dispersing medium. It is far otherwise in the case of the envelope of *Past. pestis*. Here the difficulty is to strip off the envelope without using vigorous physical or chemical procedures which are likely to produce a breakdown of its complex structure. The use of heat is therefore inadmissible and solution in alkali, the classical method of preparing soluble antigens, is also contraindicated. There are two different approaches to this problem: disintegration of the whole cell with subsequent fractionation of the complex colloidal systems resulting, or differential solution of the envelope leaving the cell otherwise intact.

Methods involving disintegration of the bacterial cell were ultimately abandoned in favour of a procedure which depended upon the use of a selective solvent. The gelatinous nature of the envelope, clearly shown by the microscope, suggested that the best approach to the problem was to study the physical rather than the chemical properties of the envelope substance. Viewed from this angle, the envelope can be regarded as a lyophilic colloid; it should therefore show imbibition swelling and, ultimately, colloidal dispersion in solutions of certain inorganic salts. Potassium iodide and KSCN, which occupy a position almost at the end of the Hofmeister series of anions, are known to possess this solubiiizing property to a marked degree. In addition, many organic substances in aqueous solution have similar properties, for example urea (Chodat, 1925; Cook and Alsberg, 1931) and sodium salicylate (Cook and Rose, 1934; McCalla and Rose, 1935).

Neuberg (1916) in an early work gave a list of more than 30 substances which have what he designates as hydrotropic properties, bringing about the solution on dispersion of substances which normally have a low solubility in water. Many of these compounds were accordingly tested as solvents of the envelope of *Past. pestis*. The results of preliminary tests agreed with the theory outlined above; imbibition and dissolution did in fact occur, and the process could be controlled without difficulty. Sodium salicylate, KSCN and KI were found to be particularly suitable, and these were accordingly studied in detail. Extraction was allowed to take place at 37° or occasionally at 45° but never at a higher temperature. The process could be followed by periodic examinations of the bacterial cells in Indian ink preparations, and was stopped as soon as solution of the envelope appeared to be complete.

The microscopic changes accompanying solution of the envelope can be described as follows. The example refers to cells suspended in 0·5 M KSCN and maintained at 37° C. During the first hour there is a perceptible widening of the space between the ink and the cell membrane, amounting sometimes to a 50 per cent increase. This is interpreted as the result of imbibition swelling of the envelope colloid. During the second hour the interface between the ink and the envelope becomes less distinct and the carbon ink particles begin to penetrate the envelope, losing as they do so their active Brownian movement and becoming sluggish or even completely immobile. The ink immediately outside the dissolving envelope now shows the appearance of an indistinct halo. This is believed to be due to the scattering of the light by the high concentration of dissolved (colloidally dispersed) envelope substance. This halo gradually disappears as the dissolved envelope substance diffuses into the surrounding medium. In this manner the zone normally occupied by the envelope progressively decreases in width until the ink particles finally reach the bacterial cell wall. The latter, no longer demarcated by the envelope, is difficult to recognize, the presence of the cell being betrayed only by a small empty space in the film of ink. The fact that the cell itself remains intact is, however, readily demonstrated by examining in the dark-field microscope a preparation made without addition of ink, or by staining the washed bacterial deposit after centrifugation to remove the solvent.

At this stage a second phenomenon is observed. The cells which have been stripped of their envelopes begin to flocculate into small clumps to which the ink particles soon become adherent. This is presumably due to the fact that the normal electric charge on the bacterial cell is removed simultaneously with the envelope. In consequence the potential falls below the critical value and spontaneous agglutination is the result. Removal of a surface antigen is well known to produce a decrease of suspension stability in weak electrolytes, hence the salt sensitivity of rough variants. The floccules remain small and discrete and precipitation does not occur spontaneously, although this can be readily brought about by light centrifugation. The appearance of the cells at different stages of solution of the envelope is shown in Fig. 7 and 8.

The optimal concentration of thiocyanate for solution of the envelope is M/2. Stronger concentrations cause both the ink and the cells to flocculate by salt action, while weaker ones are slower in action. With KI it is necessary to prolong the period of extraction to 12 or even 24 hours at 37° before solution of the envelope is complete. Sodium salicylate on the other hand is a much more active reagent, quickly removing protein from the cells as well as the envelope and causing

a marked increase of viscosity and separation of a stringy precipitate of nucleoprotein, features that are absent in the case of the other two solvents. Thiocyanate is therefore regarded as the most suitable solvent, although the effect of salicylate at different concentrations and temperatures has not yet been fully investigated.

The scale of the experiments was now enlarged in order to obtain sufficient material for chemical and immunological analysis. Both solid and fluid media were used. The former produce a considerably greater yield, but have the objection that agar may occur as an impurity in the final antigen solutions. The use of nutrient broth overcomes this difficulty, but at the expense of a lower yield of organisms. The following description of one typical preparation will serve to illustrate the methods employed:

Preparation TJW 9.ii.50.

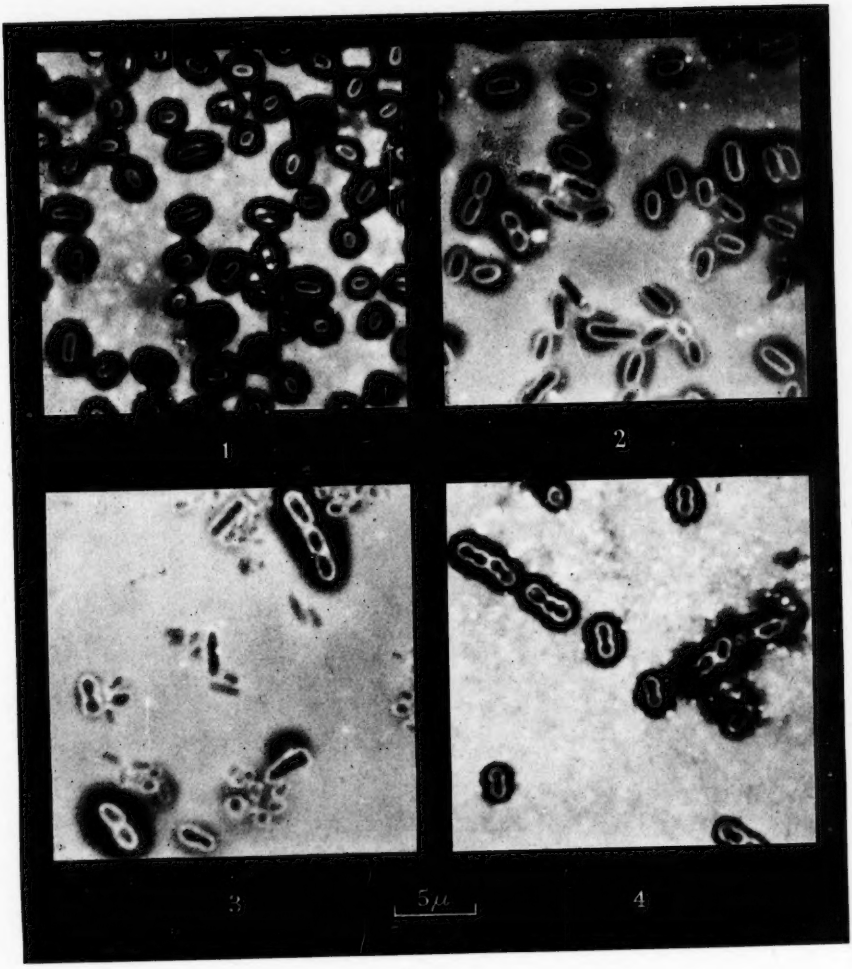
The growth from 6 culture trays was suspended in 850 ml. of distilled water. One ml. of this suspension contained 35,500 million organisms or 8 mg. of dry bacterial substance as determined by evaporating a 10 ml. sample *in vacuo* over P_2O_5. To this suspension was then added 850 ml. of M/1 KSCN solution, and the reaction was adjusted to pH 7·8 with 10 per cent KOH solution. Very little alkali was required. The mixture was held in a water bath at 37° with vigorous agitation supplied by an electric stirrer. Small additions of alkali were made at intervals in order to maintain the pH between 7·6 and 7·8. Solution of the envelope took place according to the description given above and was complete in 5 hours. At the end of this period the suspension was quickly cooled to room temperature and the reaction was adjusted to pH 7·0. There was no appreciable increase of viscosity, and no precipitate formed when the acid was added. The suspension was then passed through a Sharples centrifuge to remove the bacterial cells. The effluent, a clear straw-coloured fluid, was filtered through a Mandler candle to ensure removal of the cells. Finally, the KSCN was removed by dialysis against tap-water.

Preliminary tests showed that these crude preparations of the envelope substance were effective immunizing agents, and that they reacted to a high titre in precipitation tests with anti-plague sera. Two doses each of 0·5 ml., given subcutaneously to multimammate mice, regularly protected the animals against a subsequent challenge of 100 M.I.D. of a highly virulent strain of *Past. pestis*. The protocol of one of these experiments is given in Table I. No toxic effects attributable either to the thiocyanate or to plague endotoxins were observed with this dosage. The precipitin titres were of the order of 1 in 100,000 to 1 in 500,000.

DESCRIPTION OF PLATES.

Photomicrographs of wet Indian ink preparations illuminated by a dark-field condenser. The capsules appear as dark areas surrounding the bacterial cells. The background appears greyish-white owing to the reflection of light from the carbon particles, which do not give clear images owing to Brownian movement.

FIG. 1.—*Past. pestis*, strain Tjiwidej, cultivated on nutrient agar for 24 hours at 37°.
FIG. 2.—*Past. pestis*, strain V123 (virulent). Same cultural conditions.
FIG. 3.—*Past. pestis*, strain Tjiwidej, showing a mixture of normal capsulated cells and non-capsulated variants. Same cultural conditions.
FIG. 4.—*Pneumococcus*, type III. Serum glucose broth: 6 hours at 37°.
FIG. 5.—*Klebsiella pneumoniae* (Friedländer's bacillus). Nutrient agar: 24 hours at 37°.
FIG. 6.—*B. mycoides*. Five per cent peptone broth: 24 hours at 37°.
FIG. 7.—*Past. pestis* cells after treatment for one hour at 37°. For description see text.
FIG. 8.—The same after 7 hours.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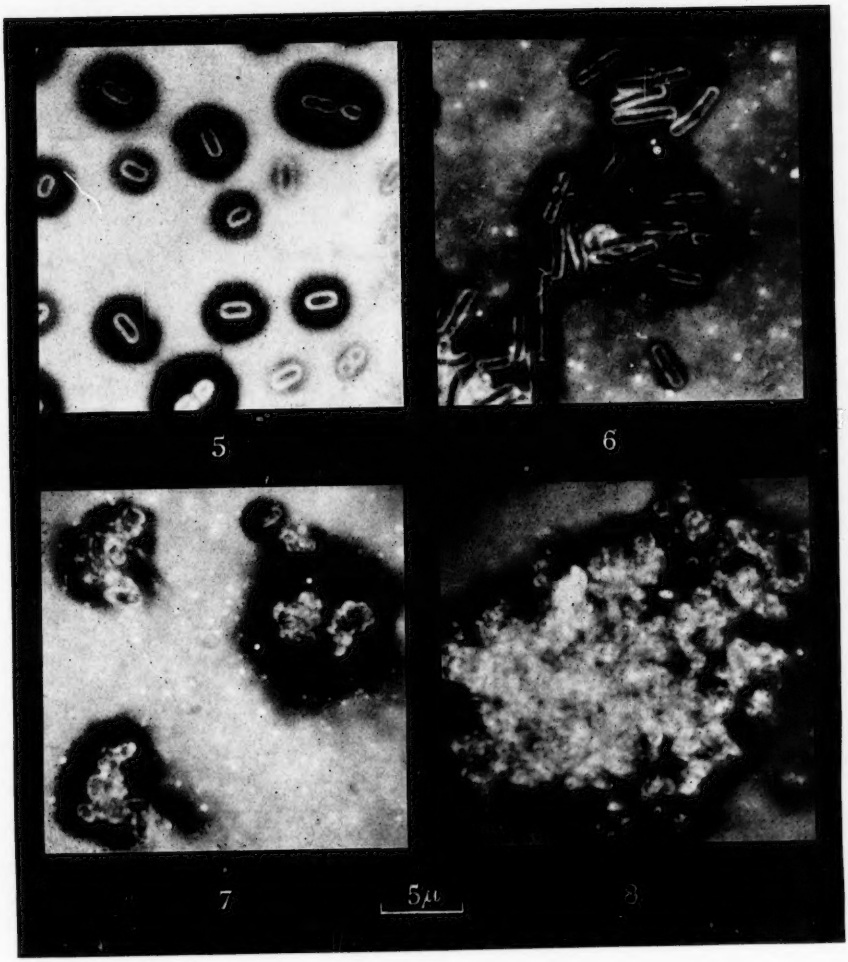
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TABLE I.—*Immunity Elicited by Crude KSCN Extracts of* Past. pestis *Strain Tjiwidej.*

Group.	Number of animals.	Dose of antigen.	Number of survivors after challenge dose of approx. 100 M.I.D. of virulent *Past. pestis.* Days									
			1	2	3	4	5	6	7	8	9	10
1	10	2 × 0·5 ml.	10	10	10	10	10	10	10	10	10	10
2	10	2 × 0·05 ml.	10	10	9	6	2	1	1	1	1	1
3	10	2 × 0·005 ml.	10	10	7	3	1	0	–	–	–	–
4	10	None. Full challenge	10	10	6	1	0	–	–	–	–	–
5	10	None. 1/10 challenge	10	10	7	3	0	–	–	–	–	–
6	10	None. 1/100 challenge	10	10	7	4	2	1	1	1	1	1

The animals used were *Mastomys natalensis* weighing approximately 45 g. Antigen given subcutaneously with 7 days' interval between injections. Challenged 7 days after 2nd dose with 700 living cells of virulent *Past. pestis* strain V123 (as determined by viable counts on blood-agar plates).

No deaths occurred after the 10th day. The survivors were killed on the 25th day; all appeared normal.

Attempted Purification of the Crude Extracts.

The crude extracts, still containing 0·5 M KSCN, remained water-clear for many months if stored at 4°. Removal of the thiocyanate by dialysis produced a slightly opalescent but still stable solution with its antigenic properties unimpaired. Filtration through a Mandler candle caused some loss of antigen. The amount of non-dialysable material (determined by evaporation *in vacuo* over P_2O_5) represented about 2 per cent of the dry weight of the organisms extracted. At this stage the usual tests for protein gave positive results, while the presence of a small amount of carbohydrate was indicated by a weakly positive Molisch reaction.

Purification was first attempted by the methods employed to isolate the soluble specific substances of the pneumococcus (Heidelberger, Kendall and Scherp, 1936; Brown, 1939). On the addition of alcohol the active material began to precipitate when the concentration of ethanol reached 40 per cent and was complete at 75 per cent. Immunization of animals with the precipitates obtained at various concentrations of alcohol showed that almost all the active material was contained in the deposit produced by 50 per cent ethanol. The flocculent precipitates were only partly soluble in water, and much of the activity remained in the insoluble residues. Repeated fractional precipitation with alcohol in the presence of sodium acetate and acetic acid caused progressive loss of activity, while the removal of the last traces of protein by shaking with chloroform and butanol (Sevag, 1934) resulted in the complete disappearance of protective antigen, although precipitins could still be detected to a titre of 1 in 80,000 with anti-plague serum. The final product represented less than 0·1 per cent of the crude extract solids after dialysis. It gave a strong Molisch reaction, but no reducing sugars were demonstrable until it had been submitted to prolonged hydrolysis with H_2SO_4. This small amount of carbohydrate was probably not derived from the medium, since it failed to react with an anti-agar serum. It was therefore considered to be of bacterial origin, analogous, perhaps, to the C substance of the pneumococcus.

A thorough trial of these methods made it clear that the activity of the complete antigen depended upon a protein constituent of the extract. The technique had accordingly to be modified in accordance with this changed concept. Some degree of purification without serious loss of activity was obtained by repeated precipitation with alcohol in the cold. The crude dialysed extract cooled to 2–4° was treated with 1·5 volumes of ice-cold ethanol. The resulting precipitate was quickly recovered by centrifugation, suspended in water at the same temperature and again centrifuged lightly to remove insoluble material. A second and third precipitation with alcohol were carried out in the same manner, the final volume being one-tenth of that of the original extract. On a dry weight basis this procedure gave a 15-fold increase of immunogenic activity. The material was therefore considered to be pure enough to justify a preliminary chemical analysis. A solution containing 5 mg. of total solids per ml. appeared as a heavily opalescent fluid with a nitrogen content (micro-Kjeldahl) of about 10 per cent of the total solids. The biuret, Millon and ninhydrin reactions gave strongly positive results, the Sakaguchi and Hopkins-Cole tests not more than a weak positive or a trace. The diphenylamine and cysteine tests for nucleo-protein (Dische, 1930, 1944) yielded completely negative results, while the Molisch and other reactions for carbohydrates were negative or very weakly positive. The presence of phosphorus could be detected in every sample. The solutions were neutral or slightly acid in reaction. They remained stable when mixed with M/5 phosphate buffer throughout the range from pH 3·0 to 11·0. In the absence of salts, however, the addition of acid (acetic or hydrochloric) produced a progressive increase of cloudiness until flocculation occurred at pH 4·8 and reached a maximum at pH 4·4. On further addition of acid the floccules dispersed but the solution remained cloudy. The precipitate at pH 4·4 contained all the protein present in the original solution and about 90 per cent of the total solids. It readily dispersed in water and dissolved completely when a trace of alkali was added to restore the reaction to neutrality. The significance of this observation was quickly realized, and isoelectric precipitation in the absence of electrolytes became the standard method of purification. It was only necessary to submit the crude extract to a thorough dialysis (tap water followed by distilled), and then to precipitate the protein by acidifying with HCl to pH 4·4, this precipitation being repeated several times.

The results of chemical tests on these purer preparations did not differ greatly from those recorded above. The reactions for protein were unchanged and phosphorus was still present, but carbohydrates could no longer be detected. No visible change took place when the neutral solutions were boiled. All the soluble material was precipitated when ethanol was added to a concentration of 60 per cent (1·5 volumes); the deposit, however, was completely soluble in water even after it had been left in contact with the alcohol overnight at room temperature. Ammonium sulphate produced flocculation when the salt concentration reached 30 per cent of saturation; or 24 per cent if the mixtures were held at room temperature for 18 hours. For these determinations a saturated solution neutralized with ammonia was used (concentration 54 g. per 100 ml. at 20°). The addition of $CuSO_4$ or $AgNo_3$ produced insoluble metallic complexes, from which the protein could be recovered by dialysis first against a citric acid buffer and then against water (Bovarnick, 1942).

In animals these preparations had approximately the same antigenic potency as those made by precipitation with alcohol. This showed that the antigen was more stable than was originally supposed. It was not inactivated by a short exposure to 60 per cent ethanol, nor was its solubility affected by a longer period of treatment with this reagent. Furthermore, it was found that the antigen could be held in a water bath at 97° (the boiling point of water at Johannesburg. for 15 minutes without any considerable loss of its immunogenic properties)

This finding, which was confirmed by a repetition of the experiment, was quite unexpected. The protocols of two representative experiments are given in Table II and III.

TABLE II.—*Immunity Elicited by Antigen Purified by Isoelectric Precipitation.*

Group.	Number of animals.	Dose of antigen.	Number of survivors after challenge dose. Days									
			1	2	3	4	5	6	7	8	9	10
1	10	2 × 500 μg.	10	10	10	10	10	10	10	9	9	9
2	10	2 × 100 μg.	10	10	10	9	8	8	8	8	8	8
3	10	2 × 10 μg.	10	10	10	7	7	4	4	4	4	4
4	10	2 × 1·0 μg.	10	10	10	5	3	3	3	2	2	2
5	10	2 × 0·1 μg.	10	10	10	5	4	4	4	4	4	4
6	10	None. Full challenge	10	10	7	3	0	–	–	–	–	–
7	10	None. 1/10 challenge	10	10	10	2	1	1	0	–	–	–
8	10	None. 1/100 challenge	10	10	10	4	0	–	–	–	–	–

The procedure was the same as that shown in Table I. The challenge dose contained 900 living cells as determined by plate counts. No deaths occurred after the 10th day. The survivors were killed on the 25th day: all appeared normal.

TABLE III.—*Comparison of Heated and Unheated Antigen.*

Group.	Number of animals.	Antigen.	Number of survivors after challenge dose. Days												
			1	2	3	4	5	6	7	8	9	10	11	12	13
1	12	Unheated. 2 × 0·5 ml.	12	12	12	12	12	12	11	10	9	8	7	7	7
2	12	Heated. 2 × 0·5 ml.	12	12	12	12	10	10	10	10	10	10	10	8	7
3	12	None. Full challenge	12	12	11	8	3	1	0	–	–	–	–	–	–

The procedure was the same as that shown in Table I. The challenge dose contained 1000 living cells as determined by plate counts. The antigen in this experiment had been prepared by the alcohol precipitation method. The heat treatment consisted of holding the sample of antigen in a boiling water bath (97°) for 15 minutes. No deaths occurred after the 13th day. The survivors were killed on the 25th day; all appeared normal.

The available evidence seems to justify the conclusion that the capsular antigen of *Past. pestis* is a relatively simple protein. However, the presence of phosphorus is not in agreement with this view, and further investigation is required to determine whether this phosphorus is an integral part of the antigen or merely an impurity. It is apparently not derived from nucleoprotein, since the tests for the latter gave uniformly negative results.

No mention has so far been made of the effect of KSCN on other capsulated bacteria. This wider field of investigation was explored to a limited extent, using bacteria of which the chemical constitution of the capsule was already known. The capsule of the pneumococcus could be clearly demonstrated by the Indian ink technique (Fig. 4). This organism, however, proved unsuitable for study for two reasons: the strains employed (mostly belonging to Type III) rapidly lost their ability to form capsules when maintained in the laboratory; and the capsules quickly dissolved spontaneously in the suspending medium (water or physiological saline) even when autolysis did not take place. *Klebsiella pneumoniae* proved more suitable for study, since it exhibits neither of these characteristics. It has an exceptionally well-defined capsule (Fig. 5), which is

known to be a completely nitrogen-free polysaccharide (Heidelberger, Goebel and Avery, 1925). KSCN used in the same manner as for *Past. pestis* was found to have no appreciable solvent action on this capsule and the stronger solvent sodium salicylate was equally ineffective.

Attention was next directed to the aerobic spore-forming bacilli, some of which under favourable conditions are known to produce abundant capsular material while others produce very little. Those who have made a study of the capsules of this group of organisms stress the importance of using certain strains that are satisfactory in this respect. The only strain that was immediately available in this laboratory was a member of the *B. mycoides* group which was under investigation on account of its antibiotic properties against *Past. pestis*. This organism produced very little capsular material when cultivated on ordinary media. It was found, however, that an infusion broth containing 5 per cent of peptone considerably stimulated capsule production. Instead of a dry, wrinkled pellicle there appeared after 24 hours' incubation in this medium at 37° a thin greyish film which could be pulled out into long strings when touched with the platinum loop. The seed for this medium should be taken from a young actively growing culture; a spore suspension is unsatisfactory. This surface growth when examined in Indian ink preparations was found to consist of bacterial cells embedded in an exceptionally large amount of capsular material (Fig. 6). Extraction with KSCN as employed for *Past. pestis* was highly successful, but sometimes required a longer period (12 to 18 hours) for complete solution of the capsules. A polypeptide was subsequently isolated from these extracts by the methods of Ivánovics and Erdos (1937) and Bovarnick (1942). These experiments will be fully described in a separate communication. So far as the present studies are concerned, these experiments with other capsulated bacteria endorsed the belief that the envelope of the plague bacillus is a simple protein and not a protein-polysaccharide complex. Still further evidence in favour of this view was obtained by noting the effect of KSCN on a number of proteins and gums of known chemical composition. Strips of gelatine, for example, rapidly swelled and ultimately dissolved in a molar solution of this salt, whereas lens protein, vitreous humour, agar-agar, gum acacia and gum tragacanth were not appreciably affected.

Immunological Experiments.

These studies were guided throughout by the results of the protection tests, examples of which have already been given. The precipitin reaction was used mainly as a rapid method of assessing the amount of antigen present before carrying out the animal tests. In plague different species of animals do not respond equally to the same antigen, the guinea-pig being particularly difficult to immunize and the rat comparatively easy. In this respect *Mastomys natalensis* stands midway between these two and is thus comparable to man. Antigens intended to be used for human prophylaxis can therefore be assayed by protection tests on this animal.

Some early experiments with *Past. pestis* vaccines confirmed the finding of Sokhey and Habbu (1941) that the protective antigen is present in the dispersing medium rather than in the bacterial cells. This was found to apply particularly to heat-inactivated vaccines. Treatment with formaldehyde retards the slow solution of the envelope that normally takes place in vaccines prepared by the former method. The supernatant fluid obtained by centrifuging a heat-inactivated phenolized vaccine is almost as effective as the whole vaccine, whereas in a formolized one the cells retain the protective antigen as well as the endotoxins.

These facts were readily established by animal experiments combined with examination of the cells by the Indian ink technique. With a challenge dose of 100 M.I.D. the amount of purified envelope antigen required to produce an effective immunity in an adult *Mastomys* of 50 g. weight was 2 to 5 μg. A human being in proportion to his weight would require about 5 mg., so that it would be an amount well within the ability of a biological institute to prepare protective doses on a large scale. The toxic (lethal) dose of the purified antigen for *Mastomys* was about 500 mg.

As already stated, the avirulent strain *Tjiwidej* was employed as the source of most of the antigens. A few were prepared from the virulent strain V123 which was always used for the challenge. A direct comparison of antigens prepared from these two strains revealed no significant difference in the degree of protection afforded. This was completely in accord with previous experience in the production of hyperimmune sera from horses injected with killed suspensions of virulent and non-virulent strains.

Hyperimmune sera were obtained by injecting rabbits with the purified antigens. The animals responded well, and showed no evidence of hypersensitivity. Towards the end of a course of injections commencing with 2 mg. and increasing to 10 mg. twice weekly, a well-marked skin reaction was obtained by injecting 5 mg. of the antigen intracutaneously. These local reactions persisted for several days but did not proceed to necrosis. To test the sera for protective antibody 50 *Mastomys* were infected with the virulent strain (60 M.I.D.). One hour later the serum (a pooled sample from 6 animals) was given intraperitoneally in doses of 0·2, 0·05, 0·02 and 0·01 ml., 10 animals being used for each dose of serum. The survival rates for these 4 groups were 10/10, 8/10, 8/10, 2/10 respectively; 10 untreated control animals all died. These results indicated that the protective antibody content of this serum was equivalent to that of a good sample of anti-plague therapeutic serum. In another experiment of this type the protective antibody content of two pooled samples of rabbit serum was compared; in one the purified envelope antigen had been prepared from the non-virulent strain *Tjiwidej* and the other from the virulent strain V123. The results of this experiment showed that the protective properties of these two sera were approximately equal.

The Application of the Keogh Antigen Adsorption Test to the Serology of Plague.

The study of the *Pasteurella* group has long been handicapped by the lack of a simple and dependable serological technique for the assay of antibody. The agglutination test is unsatisfactory on account of the difficulty of preparing stable suspensions, and a precipitin reaction is only practicable with hyperimmune sera. An indirect method of carrying out agglutination tests has recently been described by Keogh, North and Warburton (1947, 1948) which seems likely to be of considerable value. The purified plague antigen is readily adsorbed by normal mammalian erythrocytes, and the cells thus sensitized are specifically agglutinated by anti-plague sera.

It is important to note that the haemagglutination method involves the mixing of serum from one species of animal and erythrocytes from another. This may result in agglutination or even haemolysis of the red cells by anti-species antibodies in the serum and thus produce false positive reactions. This possible

source of error is avoided by removing the anti-species antibody by a preliminary absorption of the serum with erythrocytes of the species to be used in the test proper. This precaution was always observed in carrying out the test with plague antisera. Apart from this, the technique was essentially the same as that described by Keogh, North and Warburton. Both rabbit and horse anti-plague sera were employed, and the red cells were obtained from human, sheep and guinea-pig blood. The standard procedure was to use sheep cells. The preliminary absorption was carried out by adding one volume of packed cells to 10 volumes of inactivated serum and centrifuging one hour later. A 0·1 per cent solution of the purified envelope antigen was added to another portion of the erythrocytes in the proportion of one volume of packed cells to 10 volumes of antigen. After one hour's contact at room temperature the cells were collected by centrifugation and washed 3 times in physiological saline. The agglutination test was then performed by adding 0·1 ml. of the packed sensitized cells to 1·0 ml. of serum in a dilution series from 1 in 10 to 1 in 10,000 or higher. The tubes were held in a water-bath at 37° for one hour and then placed overnight in the refrigerator, the results being read on the following morning.

The haemagglutination test is unlikely to be of much value as a diagnostic procedure because of the slow development of immunity in plague. It may, however, prove useful as a means of confirming retrospectively a diagnosis made on clinical grounds alone. For research purposes it is a useful method of following the development of circulating antibody in animals undergoing active immunization. The following is an example: Three young adult rabbits were immunized with a purified preparation of the capsular antigen. The dose was 2 mg. given twice weekly, at first by the intravenous route and later subcutaneously. Samples of blood were taken at weekly intervals during and after this course of immunization and also following a re-stimulating dose. The results of the haemagglutination tests are summarized in Table IV. The rise and fall of antibody titre and the response to re-stimulation followed the usual pattern. It was hoped to obtain a parallel series of bacterial agglutinin titres of the same sera, but all the *Past. pestis* suspensions prepared for this purpose showed spontaneous clumping sufficient to render the results unsatisfactory. Up to the present there has been no opportunity to apply this test to human convalescent sera.

TABLE IV.—*Response of* 3 *Rabbits to Immunization with Purified Capsular Antigen: Haemagglutination Titres of Sera.*

Sample of serum tested.		Haemagglutinin titres.		
Number of days from commencement of immunization.	Number of immunizing doses given.	Rabbit A.	Rabbit B.	Rabbit C.
0	0	0	0	0
10	3	1/20	1/20	0
17	5	1/160	1/320	0
24	7	1/160	1/320	1/20
31	9	1/320	1/320	1/40
38	11	1/320	1/640	1/40
Resting	—	—	—	—
80	—	1/20	1/20	0
82	Re-stimulating dose	—	—	—
90	—	1/160	1/640	1/10

DISCUSSION.

It is hoped that the facts demonstrated by these experiments will put an end to the controversy regarding the envelope of the plague bacillus. The difficulties arose from too great a reliance on the appearance of the organism in stained films, and could only be resolved by the application of several different methods of investigation. Visual examination of the living bacteria in Indian ink (a method free of artefacts) removes all doubts about the reality of the substance. Sokhey (1940) has given his opinion that " the envelope appears to be nothing but an unstained capsule plus a halo which is produced by a peculiar settling down of the fine particles of the Indian ink at a distance from the capsule due to the operation of physical forces." This suggestion is quite incompatible with the physical chemistry of small particles, nor is it borne out by the observations recorded in the present paper. In fluid preparations the Indian ink particles are seen in active Brownian movement at an interface between an aqueous phase and a semi-solid phase. There is no need to postulate the existence of an " envelope " *and* a capsule, nor is there any experimental evidence to support such a contention. The substance covering the cell is simply a well-developed bacterial capsule, and it should be so named. In appearance it resembles the capsules of other bacteria, and even in size it is not exceptional, since other bacteria, for example, *K. pneumoniae*, surpass *Past. pestis* in this respect. Loss of the capsule, either as the result of spontaneous variation or deliberately produced by extraction with a solvent, leaves a naked cell uncovered by any extracellular substance. Moreover, removal of the capsule by either of these processes deprives the cell of its main immunogenic antigen.

Rowland's Indian ink dark-field technique was invaluable in the present studies and certainly merits wider application. It provides a simple means of assessing the capsule-producing properties of a strain, and any deterioration in this respect, resulting either from spontaneous variation or from growth in unsuitable media, can be readily detected. Failures to obtain soluble antigens are often due to the use of unsatisfactory starting material. The further advantage of being able to follow the process of solution of the capsule by a visual method is obvious.

No claims are made regarding the purity of the antigens prepared by these methods. This matter must await the results of a full chemical and physical analysis, which, unfortunately, could not be undertaken in this laboratory. Enough has been done, however, to suggest that the final product is a simple protein. In this respect it differs from the Fraction 1A antigen of Meyer and his associates (Baker, *et al.*, 1947), which is stated to be a protein-polysaccharide complex. At this stage, however, it would be unwise to attach much importance to this apparent discrepancy. In view of the approximately equal potency of the two antigens it seems improbable that they differ radically in the chemical constitution of the active constituent. The presence of slight impurities in one or both could account for the differences in chemical behaviour.

The advantages of using an aqueous solvent to remove bacterial capsules are shown by these experiments. This technique has the merit of simplicity ; it avoids the use of drastic procedures likely to cause chemical and physical changes in the capsular substance ; and, in the case of KSCN, the antiseptic effect of the solvent is a further recommendation for its use. It is not unlikely that further

investigation will disclose suitable aqueous solvents for capsules not susceptible to the action of those already tested. This is a promising field for research.

SUMMARY.

The so-called envelope of *Past. pestis* is nothing more than a particularly well-developed bacterial capsule. This conclusion was reached by studying the capsule in wet Indian ink preparations illuminated by a dark-field condenser (Rowland's method).

The capsular substance can be removed from the cells by certain aqueous solvents such as potassium thiocyanate. The one important immunogenic antigen of *Past. pestis* is thus obtained in a soluble form, and it can be concentrated and purified by the usual physico-chemical methods.

The final product appears to be a simple protein. It is strongly antigenic, a few micrograms being sufficient to induce a high level of immunity in experimental animals. The chemical characterization of the purified antigen has, however, not yet been determined, and until this is done no claims can be made regarding the degree of purity achieved.

The capsules of the pneumococcus and *K. pneumoniae*, which are composed of polysaccharide, are not soluble in potassium thiocyanate, whereas the polypeptide capsule of *B. mycoides* can be extracted by this method.

The methods employed hold promise of wider application in the field of immunochemistry and bacterial technology.

Some of the bacterial strains used in this investigation were kindly supplied by my colleagues, Dr. David Ordman, Dr. J. H. Mason and Mr. G. H. Ford. I have also to thank Mr. F. A. Brandt for his advice on many details of photographic technique.

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THE EFFECT OF CORTISONE UPON SOME EXPERIMENTAL HYPERSENSITIVITY REACTIONS.

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In a previous paper (Smith and Humphrey, 1949) it was shown that administration of sodium salicylate partially protected rabbits and guinea-pigs against passive Arthus reactions, and inhibited the Shwartzman reaction in rabbits, although it conferred no significant protection against anaphylaxis in the guinea-pig, nor could specific anti-histamine action be demonstrated. We were of the opinion that the increase of capillary permeability that occurs in such hypersensitivity reactions was inhibited, but were unable at the time to suggest any mechanism whereby sodium salicylate exerted its effects. The possibility that these effects were mediated through the adrenal cortex prompted an examination of the action of cortisone in comparable conditions. A number of papers have already been published demonstrating that cortisone or adrenocorticotrophic hormone (ACTH) will diminish hypersensitivity reactions, in for example tuberculous guinea-pigs (Long and Miles, 1950), and in sensitized humans (Long and Favour, 1950; Carey, Harvey, Howard and Wagley, 1950). The results of published experiments upon the Arthus reaction in rabbits are, however, somewhat contradictory. Harris and Harris (1950) state that in actively sensitized animals treated with cortisone or ACTH the reaction was not diminished, whereas Germuth and Ottinger (1950) contend that in treated rabbits it was almost abolished, although passive Arthus reactions could still be elicited equally well in treated and untreated animals. Germuth and Ottinger attributed the low sensitivity of the treated animals to their diminished antibody production. Work on anaphylactic shock is also contradictory. Harris and Harris (1950) state that anaphylaxis in actively sensitized guinea-pigs is not affected by cortisone, whereas in adrenalectomized rats and mice, which are rather susceptible to anaphylactic shock, cortisone appears to confer a definite degree of protection (e.g., Dews and Code, 1951). It seems likely, in view of the quite clear-cut results to be described, that these apparent contradictions may be due to species variation and to dosage and duration of treatment. As regards the Shwartzman reaction, both Soffer, Shwartzman, Schneierson and Gabrilove (1950) and Thomas and Mogabgab (1950) agree that ACTH or cortisone given before the provocative intravenous dose of toxin prevents the subsequent development of haemorrhages. A confirmatory experiment on the action of cortisone upon the Shwartzman reaction is included in this paper, because a technique was used which may have some advantages of convenience over that previously employed.

EXPERIMENTAL.

The Effect of Cortisone upon Passive Arthus Reactions in Rabbits.

The reversed passive Arthus reaction provides the method of choice for quantitative study (Smith and Humphrey, 1950). Two antibody systems were used: (1) rabbit antiserum prepared against crystallized bovine serum (Armour) with

the aid of Freund's adjuvant mixture (Freund and McDermott, 1942), and (2) rabbit antiserum prepared against electrophoretically pure human γ-globulin with the same mixture. The amount of specifically precipitable antibody nitrogen was determined, and the concentration of serum was adjusted so that 0·5 ml. contained 0·2 mg. antibody nitrogen. In the tests, antigen (2·0 mg. of nitrogen) was injected intravenously into sandylop rabbits weighing 2·5–2·8 kg., and 30 min. later 2 injections of 0·5 ml. antiserum were made into the skin of the belly, which had been epilated on the previous day. At suitable intervals of time (Fig. 1) thereafter the dimensions of the resulting oedematous areas were

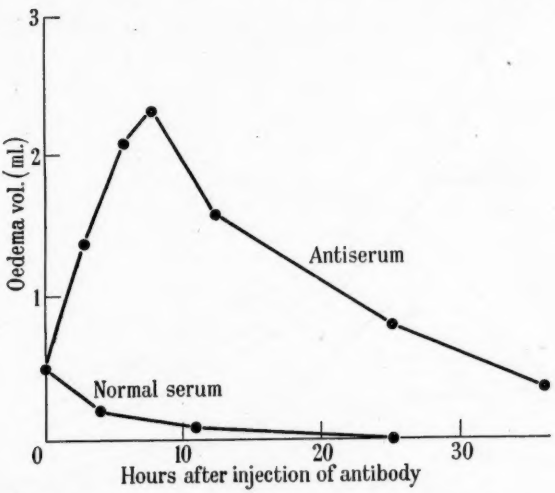

FIG. 1.—Time course of oedema at the sites of intracutaneous injection of normal serum and of serum containing 0·2 mg. antibody N.

measured and the mean volume calculated as 5 $\pi/8$ (area $\times$ height), which was found to represent fairly accurately the volume of experimental blebs produced by injecting known volumes of 0·9 per cent NaCl solution intracutaneously. Two days later the process was repeated, 1·2 mg. antigen nitrogen being injected intravenously, followed by intracutaneous antiserum at sites chosen to eliminate position variation compared with the first test. The justification for repeating the tests after two days is provided by the fact that the effect of a single dose of cortisone is no longer apparent after 36 hr. (Fig. 2). In certain experiments a third test was performed in the same way as the second after another two days. Three such tests in succession could be made in any one animal without marked variation in response, but if further tests with the same antigen-antibody system were attempted the response was diminished, presumably because the animal had begun to produce its own antibodies. The time relations of an Arthus phenomenon produced in this manner are illustrated in Fig. 1, in which antiserum and normal serum are compared.

Cortisone was administered by intramuscular injection of crystalline cortisone acetate (Merck) into the thigh, followed by brief massage of the injection site.

In Fig. 2 is shown the effect of antiserum injected into different sites on the same animal at varying intervals of time after a single dose of cortisone 5 mg./kg. Since a considerable excess of circulating antigen was present, and since site to site variation is small if care is taken to avoid areas overlying bone, the sizes of the reactions are strictly comparable, and demonstrate that cortisone in this dosage markedly prevents oedema formation and that its effect persists for at least 24 hr. In the light of this and other experiments it was decided in later tests to allow an interval of 1–1½ hr. to elapse between injection of cortisone and subsequent inoculation of antibody into the skin.

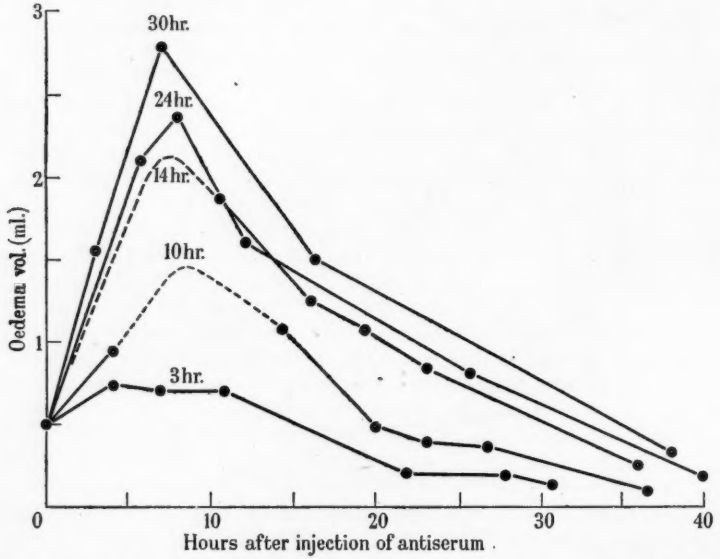

FIG. 2.—Oedema at sites of reversed passive Arthus reactions performed in the same rabbit. Antibody injected at 3, 10, 14, 24 and 30 hr. after a single administration of cortisone acetate 5 mg./kg.

An experiment was made, using rather large doses of cortisone (13 mg./kg.), to determine whether the size of the reaction in the rabbits was affected by previous tests or by previous administration of cortisone. The results of this test are shown in Fig. 3 ; treatment with cortisone greatly decreased the reaction in whichever period it was given. The size of the reactions in untreated animals in different tests was approximately constant, but there was a suggestion that after the cortisone effect had worn off the reactions were increased. Such an increase was not observed with smaller doses of cortisone (5 mg./kg. or less), but nevertheless, in subsequent tests one half of the animals were given cortisone in the first period and one half in the second in order to eliminate possible bias due to order of treatment. A series of tests was made in which the development of oedema at sites of reversed passive Arthus reactions was compared in the same rabbits with and without cortisone, at different dose levels of the drug. The results are given in Table I, in which for convenience of presentation only the

oedema volume at 8 hr., at which it is about maximum, is recorded. Doses of 13, 5 and 2 mg./kg. were almost equally effective. At 0·5 mg./kg. the effect was noticeably less, some rabbits showing a marked and others a small response, whereas with higher dosage the response was uniform.

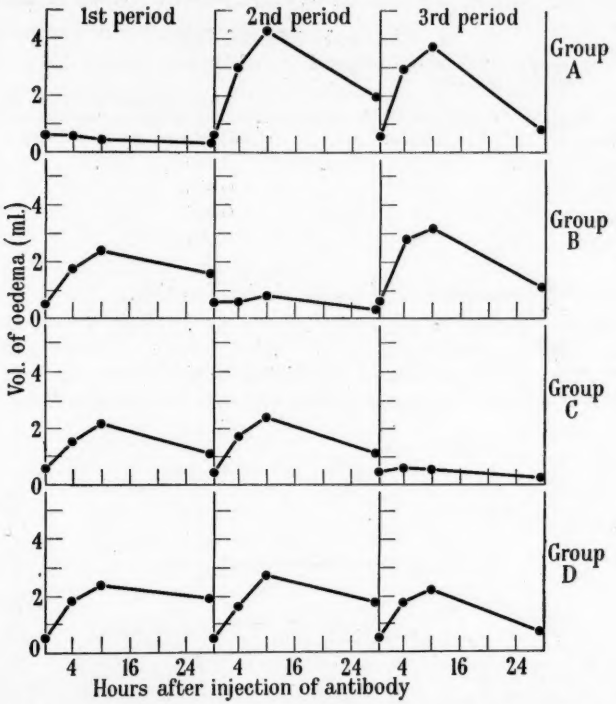

FIG. 3.—Oedema at sites of reversed passive Arthus reactions repeated at 2-day intervals. Cortisone 13 mg./kg. was administered during one period only in each group: Group A in 1st, group B in 2nd, and group C in 3rd period. Group D received no cortisone.

TABLE I.—*Effect of Cortisone on Reversed Passive Arthus Reactions.*

Dose of cortisone acetate (mg./kg.).	Number of rabbits.	Mean oedema vol. (ml.) at 8 hr.	
		Cortisone period.	Control period.
13	6	0·57	3·10
5	3	0·15	1·7
2	3	0·72	2·5
0·5	6	0·89	1·87

Local reversed Arthus reactions.

Since it was possible that the diminution of the Arthus reaction as elicited in the cortisone-treated animals was due to failure of the antigen to pass from the

circulation into the tissue fluids at the sites of antibody injection, both antigen and antibody were injected into the same sites of the skin. In 4 animals antibody was injected first, and in 4 animals antigen, the injections being separated by ½-hr. intervals. Here again (Table II) cortisone was effective.

TABLE II.—*Effect of Cortisone on Local Passive Arthus Reactions.*

A = Antibody injected first.
B = Antigen injected first.

	Dose of cortisone acetate (mg./kg.).	Number of rabbits.	Mean oedema vol. (ml.) at 6 hr.	
			Cortisone period.	Control period.
A	5 mg.	2	0·40	1·1
	2 mg.	2	0·48	1·1
B	5 mg.	2	0·35	1·3
	2 mg.	2	0·68	1·05

The effects of colchicine and ACTH on the Arthus reaction.

Colchicine was chosen as an agent known to produce a well-marked stress phenomenon (Leblond and Segal, 1938; Selye, 1950). It was administered as a single intramuscular injection of colchicine sulphate 0·6 mg./kg., which is a large dose, but insufficient to cause obvious illness in the rabbits. Two experiments

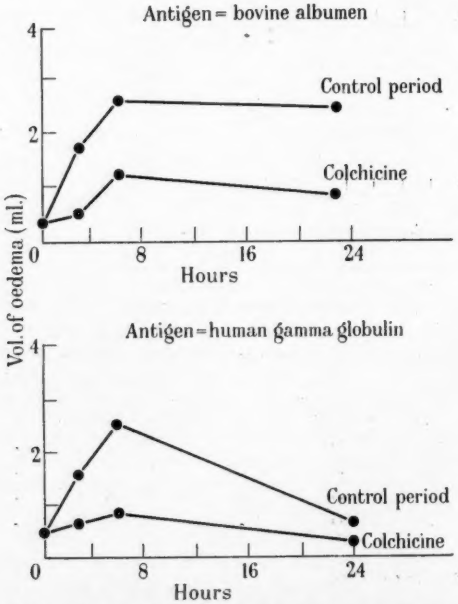

FIG. 4.—Effect of colchicine (0·6 mg./kg.) upon oedema in reversed passive Arthus reactions, using different antibody systems.

were made along the same lines as those with cortisone. In one the antigen was bovine serum albumin, and in the other human γ-globulin. The results (Fig. 4) demonstrate that colchicine in large doses notably decreased the reaction by about the same amount as 0·5 mg./kg. cortisone. ACTH was tested in two rabbits only; oedema formation was almost completely prevented by a dose of 3 international units/kg.

Further observations on the Arthus reaction.

When reversed Arthus reactions are evoked in rabbits which have received a previous intravenous injection of Pontamine Sky Blue 6 Bx (30 mg./kg.), the sites at which antibody was injected become stained blue after $\frac{3}{4}$–$1\frac{1}{2}$ hr. During

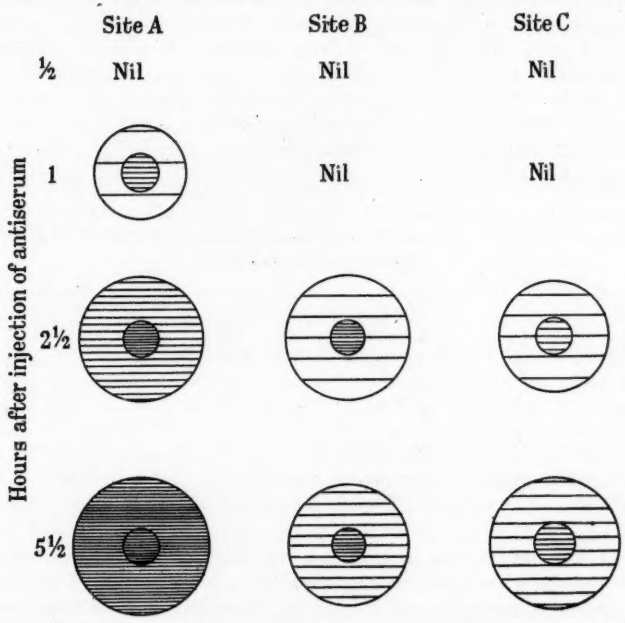

FIG. 5.—Diagram to illustrate the time course of blueing at the site of reversed Arthus reactions performed in the same rabbit, previously injected with Pontamine Blue. Antiserum was injected at site A 1½ hr. before, at site B 1 hr. after, and at site C 2 hr. after administration of 5 mg./kg. cortisone. The shading is proportional to the intensity of blue.

the subsequent 4 hr. the central area becomes more intensely stained, and the blueing spreads peripherally. In rabbits in which generalized blueing of the untreated skin did not occur for 8 hr. or more, it was possible to observe reactions at corresponding sites in the same animal before and after administration of cortisone. Fig. 5 summarizes one such experiment, and it will be seen that after cortisone the central blue area is less intense, and the peripheral spread is almost absent.

Histological examination was made of the sites of reversed Arthus reactions in cortisone-treated (5 mg./kg.) and normal rabbits. Three injections of rabbit

antiserum and three of normal rabbit serum were made in each animal, and the skin sites excised under local anaesthesia 2½, 5 and 8 hr. after injection. Moderate diffuse infiltration with polymorphonuclear leucocytes occurred at the sites injected with normal serum, and intense infiltration, particularly around the vessels, occurred in the presence of antiserum. The only differences observed between cortisone-treated and control animals were that in the treated animals there was less oedema, and that the infiltration with polymorphonuclear leucocytes was less marked both at the normal and the antiserum sites. Qualitatively the changes were the same.

The effect of cortisone on anaphylaxis in guinea-pigs.

In order to ensure that the animals should be uniformly sensitized they were passively sensitized by intravenous injection of rabbit antibovine albumin serum. The doses used were 0·16 mg. and 0·2 mg. antibody N per kg., which had been found in previous experiments to give nearly 100 per cent mortality from anaphylactic shock when 2·0 mg. antigen was injected intravenously two days later. The guinea-pigs were shocked 5–6 hr. after receiving 5 mg./kg. cortisone acetate intramuscularly. This dose was found by Long and Miles (1950) to produce maximal inhibition of allergic response to tuberculin. The results (Table III)

TABLE III.—*Effect of Cortisone upon Anaphylactic Shock in Passively Sensitized Guinea-pigs.*

Mg antibody N per kg.	Number of animals.	Dose of cortisone (mg./kg.).	Mean anaphylactic score.
0·16	6	5	1·7
0·16	6	—	4·2
0·2	6	5	3·2
0·2	6	—	5·0

The anaphylactic score was assessed as follows:

Death in 10 min. = 5; death after 10 min. = 4; severe shock = 3; moderate shock = 2; mild shock = 1.

show that cortisone-treated animals were slightly but definitely protected, particularly in the group which received the smaller dose of antiserum. The degree of protection, however, was very much less than that afforded by 10 mg./kg. doses of the anti-histamine drug "Anthisan" (pyranisamine maleate).

Cortisone and the Shwartzman phenomenon.

The Shwartzman phenomenon was produced by means of a complex polysaccharide isolated from cultures of *S. marcescens* by the method of Hartwell, Shear, Adams and Perrault (1943–44). Haemorrhagic lesions in rabbits were fairly regularly produced by intracutaneous injections of 20–40μg., followed by intravenous injection of a similar dose after 24 hr. It was observed that rabbits in which this procedure was repeated at approximately 10-day intervals responded rather regularly, despite the production of precipitating antibodies against the polysaccharide. Hence it was possible to reduce the number of animals required for testing the effect of cortisone by interposing a test with cortisone between

tests in which no hormone was given. Cortisone acetate 6 mg./kg. was injected 1½ hr. before the provocative intravenous injection of polysaccharide, and the area of haemorrhage and the height of the lesion above the surrounding skin was measured after 5 hr. The results given in Table IV show that cortisone inhibited the development of haemorrhage. More striking though less readily tabulated was the diminution of thickening of the skin at the reaction sites.

TABLE IV.—*Effect of Cortisone upon Shwartzman Phenomenon.*

Number of test.	Dose of cortisone (mg./kg.).	Mm. haemorrhage at skin site injected with 20 μg. polysaccharide. Rabbit number. 39	40	41	42
9th	*nil*	15×15	25×25	20×20	10×10
10th	6	trace	trace	very faint, 15×10	—
11th	*nil*	15×10	20×10	22×15	18×10

Colchicine sulphate 1·0 mg./kg., administered subcutaneously in divided doses, 2 hr. before the intracutaneous injection and 1 hr. before the intravenous injection of polysaccharide, did not diminish the haemorrhagic reactions, although there was some diminution of the subsequent oedema.

DISCUSSION.

This work was originally undertaken in order to ascertain whether the decrease in the Arthus and other reactions previously observed after administration of large doses of salicylate could be explained on the basis of cortisone-like action, either primarily due to salicylate itself or secondarily as a result of adrenal stimulation. From a study of the Arthus and Shwartzman reactions and of anaphylactic shock, it may be concluded that the modifications produced by salicylate palely mirror those produced by cortisone. There are other ways in which the effects of the two substances run parallel. Either substance during immunization can decrease antibody production (e.g., Swift, 1922; Jager and Nickerson, 1947; Germuth and Ottinger, 1950), and will prevent or diminish the allergic arteritis that follows large intravenous doses of foreign serum (e.g., Smull, Wissler and Watson, 1948; Rich, Berthrong and Bennett, 1950). Furthermore, I have observed that cortisone will raise about fourfold the threshold concentration at which histamine, or leucotaxine, or the histamine liberator 48–80 (Burroughs Wellcome & Co.), will cause leakage of dye at the site of injection in rabbits previously injected with Pontamine Sky Blue. This confirms the claim of Bangham (1951), and provides a further parallel with the effects of salicylate. Clinically resemblances between the metabolic effects of the two substances have been reported by Cochran, Watson and Reid (1950). Thus there is a wide resemblance between some of the effects of cortisone and of near-toxic doses of salicylate. Such doses of salicylate are stated to be high enough to cause a fall in circulating eosinophiles in mice (Kelemen, Majoros, Ivanyi and Kovacs, 1950), and it may be a sufficient explanation that salicylates cause increased adrenal cortical activity rather than that they possess cortisone-like

activity as such. Limited studies with colchicine show that a quite different substance, known to cause acute adrenal stimulation, has similar effects upon Arthus reactions. The fact that colchicine did not diminish the Shwartzman reaction weakens the analogy. The Shwartzman reaction, however, is rather a peculiar phenomenon, and appears to depend upon the level of circulating polymorphonuclear leucocytes (Stetson and Good, 1951). Colchicine, unlike cortisone or sodium salicylate, causes a marked leucocytosis (Dixon and Malden, 1908), and its effects are therefore not strictly comparable.

The experiments with cortisone on the reversed passive Arthus reaction demonstrate that single doses of 2 mg./kg. or over, and in some animals even 0·5 mg./kg., will cause a great diminution in the intensity of reactions of the severity of those studied. At none of the dose levels used were the reactions completely abolished—a finding which accords with that of Long and Miles (1950), who were able to demonstrate great reduction but not abolition of tuberculin hypersensitivity in cortisone-treated guinea-pigs. In the Arthus reaction signs of vascular damage do not appear for about 1 hr. and the reaction develops slowly. An agent which over a long period could even double or treble the resistance of the vessels to damage would greatly alter the course of the visible reaction. In anaphylactic shock in guinea-pigs, however, the acute symptoms result from overwhelming broncho-constriction, due to the release of histamine consequent upon the antigen-antibody reaction. To prevent anaphylaxis in guinea-pigs a protective agent would need to prevent the antigen-antibody reaction from taking place, or the consequent histamine release, or to have a marked anti-histamine action. There is no evidence that cortisone has either of the first two properties, or that its weak anti-histamine action is in any way specific. It is not surprising, therefore, that cortisone should not prevent death from anaphylactic shock in highly sensitized animals, and should only weakly modify anaphylaxis in animals that are less sensitized.

Finally, it should be emphasized that in this work single doses of cortisone have been employed, and that the results do not necessarily apply to animals which have had prolonged courses and in which the endocrine balance may be profoundly altered.

SUMMARY.

1. A simple cross-over technique is described whereby the effect of drugs upon the oedema at the sites of reversed passive Arthus reactions in rabbits can be measured.

2. A single dose of cortisone greatly diminishes such oedema. A dose of 0·5 mg. per kg. is effective, but greater effects are produced by 2 mg. or more per kg. The effect of cortisone is apparent within 1 hr. after injection, and persists for up to 24 hr. Similar but less marked effects upon passive Arthus reactions were produced by colchicine.

3. Administration of cortisone conferred a definite but minor degree of protection against anaphylactic shock in guinea-pigs.

4. Repeated Shwartzman reactions were produced in rabbits. Cortisone, but not colchicine, prevented the reactions.

5. A comparison of the effects of cortisone with those of large doses of sodium salicylate suggests that the effects of salicylate upon hypersensitivity reaction may be mediated through the adrenal cortex.

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ADDENDA

VOL. XXXII, No. 3, JUNE, 1951.

The following figure legends were omitted from the paper by G. T. Cook *et al.*, p. 203.

FIG. 1.—Growth of *Sh. sonnei* "normal" and variant strains, in peptone water with and without sugars. Basal medium (B.M.): 2 per cent peptone water containing M/20 phosphate buffer. Sugar: To give M/50 final concentration.

(1) "Normal" organism in B.M.
(2) " " B.M.+lactose.
(3) " " B.M.+sucrose.
(4) Lactose variant in B.M.
(5) Sucrose " "
(6) "Normal" organism in B.M.+glucose.
(7) Lactose variant in B.M. +glucose.
(8) Sucrose " " "
(9) " " " +sucrose.
(10) Lactose " " +lactose.

FIG. 2.—Growth of the lactose and sucrose variants from adapted and unadapted inocula. Basal medium (B.M.): 2 per cent peptone water containing M/20 phosphate buffer. Sugar: To give M/50 final concentration.

(1) Lactose variant in B.M.
(2) " " " +glucose.
(3) " " " +lactose (sub-cultured from agar).
(4) " " " " (" B.M.+lactose).
(5) Sucrose " "
(6) " " " +glucose.
(7) " " " +sucrose (subcultured from agar).
(8) " " " " (" B.M.+sucrose).

ADDENDA

VOL. XXXII, No. 3, JUNE, 1951.

The following figure legends were omitted from the paper by C. G. Pope *et al.*, p. 246.

FIG. 1.—The nitrogen content of the floccules for a constant amount of antitoxin (300 units) and varying amounts of toxin (data from Pappenheimer and Robinson, 1937).

FIG. 2.—The precipitation of a batch of toxin with 3·5 M potassium phosphate. This toxin showed 3 exponential steps; usually the number is greater than this.

FIG. 3.—The floccule nitrogen curves for three toxins against the same peptic antitoxin.

FIG. 4.—The diffusion test for antigen-antibody "precipitin" lines.

FIG. 5.—The proteolytic enzymes present in culture filtrate of *C. diphtheriae gravis* (CN 2026). Inhibition of enzyme activity at the points marked × by antibody serum is shown in Table IV.

ADDENDA

VOL. XXXII, No. 3, JUNE, 1951.

The following figure legends were omitted from the paper by L. B. Holt, p. 157.

FIG. 1.—Test for normality of distribution of logs. of titres.

Base-line titres following a second stimulus (Table I, col. 2).

FIG. 2.—Test for normality of distribution of logs. of titres.

Maximum second response titres (Table IV).

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(4) " " " " (" B.M.+lactose).
(5) Sucrose " "
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reduction in resistance to infection.

Experiments were therefore designed to show whether the protein-deficient diets which we were using would impair the ability of rats to form agglutinins against bacteria, and whether such diets would impair the ability of rats to form complement. It was planned to test the same animals for resistance to infection in an attempt to correlate complement or antibody titres with resistance to infection.

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(6) " " " +glucose.
(7) " " " +sucrose (subcultured from agar).
(8) " " " " (" B.M.+sucrose).

b and *c*; Glynn and Himsworth, 1944). MacCallum and Miles (1946), using a diet suggested by Himsworth, which was supposed to contain the minimum amount of casein necessary to protect rats' livers from acute necrosis for at least 60 days, attempted to pass infective hepatitis to albino rats.

In both the work of MacCallum and Miles and that of Himsworth and Glynn (Himsworth, personal communication) difficulty was experienced from the high rate of bacterial infection in rats on protein-deficient diets, and numerous experiments had to be repeated because of such casualties. In the former work rats were obtained from two different sources, and those from the one source were commonly infected with *Salmonella typhyi-murium*, while those from the other succumbed to a diphtheroid which is named *Corynebacterium murium* or *Coryne bacterium pseudotuberculosis murium* (Wilson and Miles, 1946). The latter organism was originally described by Klein (1903), and was said to have a particularly high pathogenicity for rats on inadequate diets (Mitchell, 1912).

Neither of these organisms attacks the liver predominantly, although both are likely to produce hepatic lesions, and we therefore considered the possibility that the type of diet which we were using was not just one which would make the liver more susceptible to infection, but might be associated with a more general reduction in resistance to infection.

Experiments were therefore designed to show whether the protein-deficient diets which we were using would impair the ability of rats to form agglutinins against bacteria, and whether such diets would impair the ability of rats to form complement. It was planned to test the same animals for resistance to infection in an attempt to correlate complement or antibody titres with resistance to infection.

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